Application of epidemiology and animal health economics in control of bovine leukemia virus (BLV) infection among dairy cows in Hokkaido Prefecture

Satoshi Nakada

Application of epidemiology and animal health economics in control of bovine leukemia virus (BLV) infection among dairy cows in Hokkaido Prefecture

(北海道の酪農における牛白血病ウイルス感染制御のための
疫学および家畜衛生経済学の応用に関する研究**)**

Veterinary Epidemiology

Doctoral Course of Veterinary Medicine

Graduate School of Veterinary Medicine

Rakuno Gakuen University Graduate School

Satoshi Nakada

Supervisor: Professor, Dr. Kohei Makita,

Veterinary Epidemiology Unit,

Graduate School of Veterinary Medicine

2020

Contents

Abbreviations

BLV: bovine leukemia virus

CI: confidence interval

EBL: enzootic bovine leukosis

GLM: generalized linear model

MAFF: Ministry of Agriculture, Forestry and Fisheries

PVL: proviral load

General introduction

Dairy, a major industry in Hokkaido, Japan

The industrial structure of Hokkaido Prefecture is characterized by a higher ratio of primary industry to Gross Hokkaido Product than that of the other prefectures [33]. In Hokkaido Prefecture, raw milk production accounted for about one fourth of the total prefectural agricultural production, and a half of the national milk production in Japan in 2010 [34]. The names of the main raw milk production regions in Hokkaido are Tokachi, Nemuro, and Okhotsk regions in descending order [35]. In the area of focus throughout this thesis, Nemuro region, dairy farming is the main industry.

For diary industry, infectious diseases in cattle are the critical threats. In Asia where Hokkaido is located, highly infectious disease such as foot-and-mouth disease (FMD), highly pathogenic avian influenza, and African swine fever continue to occur in livestock industries [110]. In Hokkaido Prefecture, FMD occurred in 2000 [90]. In this outbreak, fortunately the epidemic was contained at only one premises; however, it made the dairy industry aware about the risk of infectious diseases, while it was expanding the production scale by increasing the number of cows and saving labor costs. To respond to such situations, the government of Japan introduced the Standards of Rearing Hygiene Management under the Domestic Animal Infectious Diseases Control Act in 2004, keep amending every five years; and provides hygiene guidance to livestock farm owners, including early detection and notification of livestock showing abnormalities and daily disinfection [60].

In Hokkaido Prefecture, national disease control programs on Johne's disease, bovine leukemia virus (BLV), and bovine viral diarrhea-mucosal disease are in place under the Domestic Animal Infectious Diseases Control Act, and the guidelines established by the Ministry of Agriculture, Forestry and Fisheries (MAFF) are available. However, the numbers of cases of Johne's disease and enzootic bovine leukosis (EBL) are increasing every year [34]. There is a compensation for Johne's disease at the legal culling of the positive reactors, while no compensation is in place for EBL cows. For the dairy industry in Hokkaido, high numbers

of EBL and Johne's disease cases should be causing great economic damage; however, quantification of such economic losses has not been conducted.

Epidemiology

Epidemiology is defined as the study of disease in populations and factors that determine its occurrence [101]. In livestock productions, application of epidemiology in disease control has a favorable effect of reducing the costs associated with livestock diseases [59]. There is an established field of study dealing with the cost of disease called animal health economics, and this can provide economic justification for proposed control options, which usually are planed based on epidemiological considerations [78]. The research on animal health economy is quite active overseas [13, 30, 79, 100], and there are a few studies conducted in Japan as well [50], including the studies targeting Johne's disease and BLV infection in Hokkaido [74].

Thesis layout

In Chapter 1, to provide cheap and rapid method of identifying high risk cows for within and between farm spreads of BLV infection, BLV proviral load (PVL) was predicted by age and lymphocyte count, using the data from 10 dairy farms in the Kushiro-Nemuro region, Hokkaido, Japan.

In Chapter 2, the economic impact of BLV infection through carcass weight loss of dairy culled cows in Hokkaido Prefecture was assessed by applying mediation analysis, using the data from 12 farms in the Kushiro-Nemuro region. Mediation analysis evaluates the relative magnitude of different path-ways and mechanisms by which exposure may affect an outcome [104]. This study investigated the causal relationships among BLV infection status, the number of abnormal findings postmortem examination (AFPE), and carcass weight of culled dairy cows adjusted for age, lactation stage, and owner. In these tripartite relationships, BLV infection caused carcass weight loss, mediated by an increased number of AFPE due to degraded immune

status was hypothesized. Based on the latest BLV prevalence data [65], the production loss of carcass weight dairy culled cow caused by BLV infection in annual Hokkaido was estimated.

In Chapter 3, the economic impact of BLV infection from mastitis of dairy cows was assessed by survival analysis and a point estimate simulation using the data from 9 farms in the Kushiro-Nemuro region and published data. Survival analysis evaluates time to occurrence of an event [15]. This study examined the causal relationships among BLV infection status, and event (mastitis) day adjusted for parity, delivery season， owner, and cow's individual difference.

In the general discussion, results of the previous chapters and further prospects are discussed.

Chapter 1. Estimation of circulating bovine leukemia virus levels using conventional blood cell counts

1.1. Introduction

Bovine leukemia virus (BLV) induces enzootic bovine leukosis (EBL) in cattle [109]. There are three disease stages of EBL: asymptomatic or aleukemic stage (AL), persistent lymphocytosis (PL), and leukemia or lymphoma [20]. BLV infection is a global health problem in cattle that is responsible for significant financial losses in the dairy industry [7].

Several western European countries with a low prevalence of BLV infection have achieved elimination of BLV infection through national control campaigns using a “test and eliminate” strategy [75, 83]. In contrast, the prevalence of BLV infection is high in Argentina [28], Canada [69], Japan [65], and the United States [103], which do not have financial compensation policies for the disease [83]. In Japan, bovine leukosis is a notifiable disease and has been subject to passive surveillance since 1997 [65]. Since then, the number of bovine leukosis cases has continued to increase annually [55, 65]. In the latest epidemiology investigation, the seroprevalence of BLV infection for dairy cattle was 40.9% (95% confidence interval (CI): 40.4–41.4) [65]; therefore, in 2015, the Ministry of Agriculture, Forestry, and Fisheries (MAFF) of Japan published ‘Guidelines for Biosecurity Measures of Enzootic Bovine Leukosis’ [54]. In these guidelines, the “test and segregate” or “test and manage” strategy is recommended, and diagnostic methodologies such as ELISA, qualitative PCR, and quantitative real-time PCR were introduced; however, methodologies to follow were not strictly specified. Real-time PCR and ELISA are widely used in Japan to disclose BLV infected dairy cattle [72]. The “test and segregate” or “test and manage” strategy has been practiced in Argentina as well [4, 26, 27]. Quantitative real-time PCR has been used to determine proviral load (PVL) in Japan [39, 40] as a research basis, and BLV-infected cows with PL have been shown to have significantly higher PVL than AL cows [76]. Some groups suggested that cattle with high PVL are efficient transmitters [28, 76]. Although there are such advantages, real-time PCR has not been used in Japan as a uniformed routine tool in the national control program, because of the high cost and complex equipment required [72].

Researchers have proposed several alternative methods to the quantitative real-time PCR method. Serological studies suggested that antibody titer against BLVgp51, the main antigenic glycoprotein of the viral envelope, is useful in the detection of BLV-infected cattle including non-lymphocytotic ones, while anti-BLVp24 antibody titer correlates with PVL regardless the status of lymphocytosis, suggesting being a potentially useful detection tool of efficient transmitters [41, 28]. However, these studies quantified PVL per a volume of DNA, not the level of PVL per a unit volume of blood. Moreover, the other study suggested only partial correlation between PVL per 100,000 peripheral white blood cells measured using real-time PCR and serological results [40]. A hematological approach showed that white blood cell count was correlated with blood PVL, and can be a good monitoring tool [4].

Although these studies proposed less expensive and more convenient alternatives to the quantitative real-time PCR method, they have not achieved in estimation of the level of PVL per a unit volume of blood. Therefore, this study was conducted to establish a quantitative procedure for estimating blood PVL/μl of BLV infected dairy cattle using a statistical model based on conventional blood cell counts and cow age to offer a cost-effective alternative to the quantitative real-time PCR method.

1.2 Materials and methods

1.2.1. Study design

This study consists of three steps. First, the statistical relationship between blood lymphocyte count (Lym) and age among BLV non-infected cattle was studied as a baseline. Second, the statistical model to estimate PVL/μl was established using BLV infected cows. Third, verification of this statistical model was conducted using cows in the other farms.

1.2.2. Areas and farm and cow selection

For the baseline survey on the relationship between Lym and age, 444 BLV non-infected Holstein cows were studied from purposively selected five dairy farms in the Tokachi and Kushiro regions of Hokkaido, Japan. These five farms included a BLV free farm, and the other farms were known to have infected cows.

For the establishment of a statistical model, a total of 250 Holstein cows infected with BLV from 10 commercial dairy farms within the Nemuro and Kushiro regions of Hokkaido, Japan were purposively selected. One of these 10 farms participated in the baseline survey mentioned above. Table 1 shows the numbers of infected cows studied and previous BLV test results based on either PCR or ELISA in these farms. Of the 10 farms, 8 used tie stalls and 1 used free stalls. The remaining farm had both tie and free stalls. The farm size ranged between 54 and 284 cows, with a median of 141 cows. These cows were between 0.5 and 14 years old and apparently healthy, without any clinical signs of EBL. The within-farm prevalence of BLV on 10 farms ranged between 7.5% and 43.7%, and overall animal level prevalence was 25.8%.

To verify the predictability of the statistical model which used the data from above 10 farms, 92 apparently healthy but BLV infected Holstein cows from two commercial dairy farms in the Tokachi region were selected.

Table 1. Numbers of infected cows studied and previous BLV test results

Farm ID	Style	Number of cows	Previous prevalence (%)[*1]	Diagnosis used[*1]	Infected cows studied[*2]
A	Tie stall	84	3.0%	PCR	24
B	Tie stall	119	43.7%	PCR+ELISA	10
C	Tie stall	200	9.5%	ELISA	18
D	Tie stall	189	20.6%	PCR+ELISA	21
E	Free and tie stalls	229	27.5%	PCR	40
F	Tie stall	103	43.7%	PCR	20
G	Tie stall	80	7.5%	PCR	5
H	Free stall	284	32.4%	PCR+ELISA	92
I	Tie stall	67	10.4%	PCR	7
J	Tie stall	54	27.8%	ELISA	13

[*1] Initial diagnosis; [*2] Cows whose PVL was quantified using CoCoMo-qPCR

1.2.3. Sample collection

Owner consent from all selected farms was obtained in advance for collection of blood samples and age information for research purposes. Blood sampling and age information collection were conducted in the above mentioned 16 farms for all the baseline, model establishment, and verification studies between October 2014 and December 2015.

In this study, blood samples were collected by caudal or jugular venipuncture immediately injected into vacuum blood collection tubes containing EDTA-2K and kept in cold storage. The next day, whole blood was brought to the Agricultural Research Department of Hokkaido Research Organization Animal Research Center, where Lym, white blood cell count, and quantification of PVL/cell were assayed.

1.2.4. Blood lymphocyte count

Lym and white blood cell count were assayed on the same day that samples were received using an automated hematology analyzer (Sysmex XT-2000iV; Sysmex, Hyogo, Japan).

1.2.5. BLV diagnosis

BLV infection status of animals were identified based on either detection of anti-BLV antibody or BLV provirus, as this study was conducted under ordinal veterinary clinical services. Anti-BLVgp51 antibodies were detected using a commercial ELISA kit (JNC Inc., Tokyo, Japan). BLV provirus was detected by nested PCR targeting the BLV long terminal repeat region (LTR), which is described elsewhere [95].

1.2.6. Quantification of BLV PVL by BLV-CoCoMo-qPCR

Genomic DNA was isolated from EDTA-treated whole blood samples using the Wizard Genomic DNA Purification Kit (Promega, Tokyo, Japan) and quantified using BLV-CoCoMo-qPCR (Riken Genesis, Tokyo, Japan). PVL per white blood cell was quantified by evaluating

the number of copies of BLV LTR gene normalized to that of the bovine leukocyte antigen DRA gene [39]. Blood PVL/μl was quantified by multiplying the PVL per white blood cell and white blood cell count per μl.

1.2.7. Statistical analysis

Data from sampled cows were recorded and handled using commercially available spreadsheet software (Excel 2013; Microsoft Corp., Redmond, WA, USA).

Understanding the statistical relationship between Lym and age among BLV non-infected cattle

For the baseline data of BLV non-infected cattle, the relationship between Lym and age of cattle was analyzed using a Poisson regression model in Generalized Linear Model (GLM) framework with quasi-Poisson errors selecting Lym as an outcome variable and age as an explanatory variable, because of the overdispersion of Lym data [11, 12, 106].

Establishment of a statistical model to estimate the blood PVL/μl

For the establishment of a statistical model to estimate the blood PVL/μl, at first, correlations between logLym and logPVL/μl, logLym and age, and logPVL/μl and age of BLV-infected cows were tested by Spearman's rank correlation coefficient (Spearman's ρ). Logarithm of Lym and blood PVL/μl were used as logarithm of a count is known to follow Normal distribution (log-link) [11] and the tests can show more robust results than using the original scale. Second, univariable analyses were performed for BLV-infected cows using Poisson regression models in GLM framework with quasi-Poisson errors, with blood PVL/μl as an outcome variable and logLym and age as explanatory variables, respectively. Third, multivariable analysis was performed using a Poisson regression model in GLM framework with quasi-Poisson errors, with blood PVL/μl as an outcome variable and logLym, age, and their interaction as explanatory variables.

Understanding the statistical relationship between Lym and age among BLV non-infected cattle

To evaluate the predictability of the statistical model, the correlation between the measured blood PVL/μl and blood PVL/μl estimated by the statistical model was tested using Spearman's rank correlation coefficient. In addition, although blood PVL does not follow Normal distribution, linear regression was performed to show the predictability of the proposed statistical model. Moreover, Spearman's rank correlation tests were performed between logPVL/cell and log of white blood cell count, and between logPVL/cell and age, to explain the model predictability.

All statistical analyses were performed using R version 3.2.2 [97] and R studio 1.2.5042 [96].

1.3. Results

1.3.1. Descriptive statistics

For BLV non-infected cows, the median and mean age were 2.60 and 2.96 years (2.5 and 97.5 percentiles: 0.50, 7.99), and the median and mean Lym were 4035.0 and 4421.0 cells/μL of blood (2.5 and 97.5 percentiles: 2150.8, 8809.3), respectively. In contrast, the median and mean age of BLV infected cows studied were 4.88 and 5.10 years (2.5 and 97.5 percentiles: 0.90, 11.38), and the median and mean Lym of these cows were 5,075.0 and 6,328.5 cells/μL of blood (2.5 and 97.5 percentiles: 1,826.8, 19,755.5), respectively.

The median and mean blood PVL of BLV infected cows were 2,274.2 and 5,020.9 copies/μL (2.5 and 97.5 percentiles: 0.3–25, 279.0), respectively. Provirus was detected from all the 154 ELISA positive samples used for statistical modelling (farms B, C, D, H, and J in Table 1), except one non-lymphocytotic cow diagnosed BLV positive using both ELISA and nested PCR but had a PVL of 0 copies/μL, and this cow was excluded from following statistical analysis due to the contradiction of PCR results.

1.3.2. Relationship between Lym and age among BLV non-Infected cows epidemiology

Figure 1 shows the relationships between Lym and age among both BLV infected, and non-infected cows. A decline of Lym over age was observed among non-lymphocytotic cows regardless the infection status, and the baseline study GLM result among BLV non-infected cows showed significant negative linear relationship between logLym and age (slope $= -0.16$, standard error: SE $= 0.01$, $p < 0.01$).

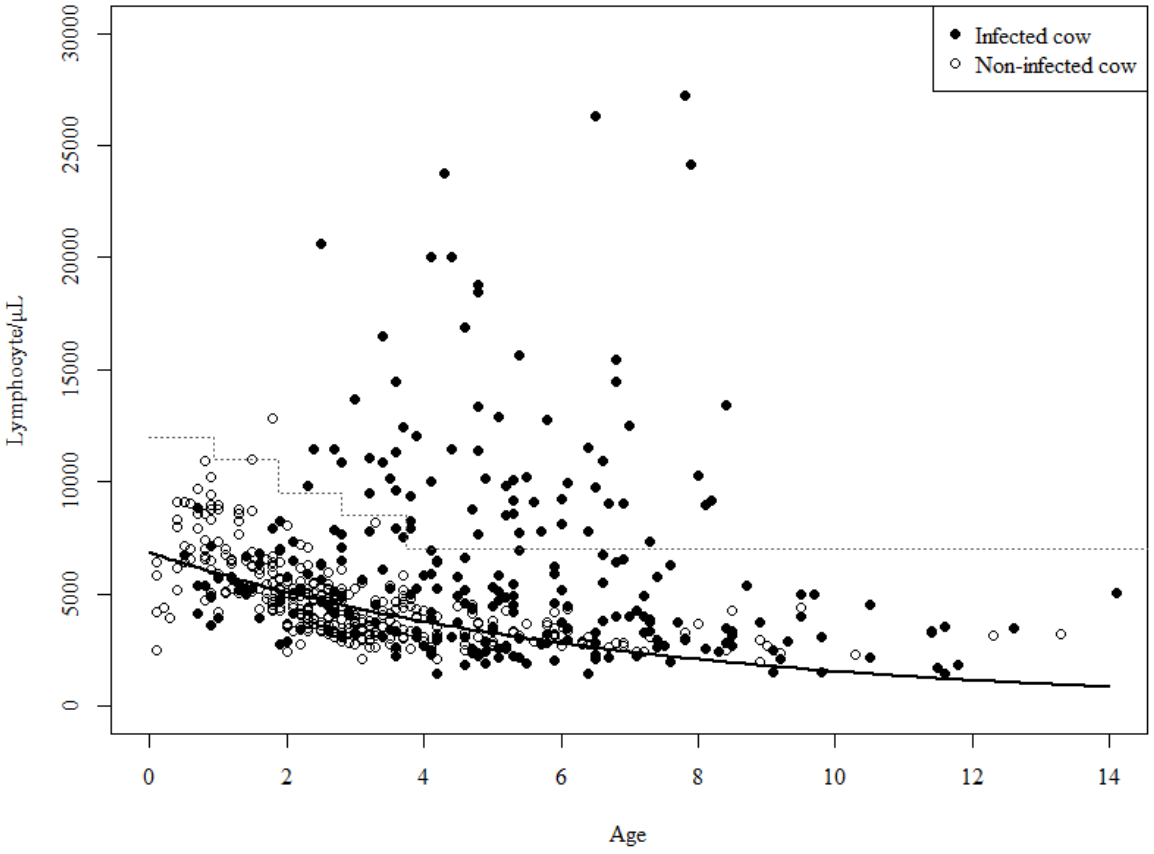

Figure 1. Scatter plot showing the relationships between Lym and age of non-BLV-infected and infected cows. A solid line shows predicted line by the model for non-BLV-infected cows, and dotted line the key of European Community.

1.3.3. Prediction Model

As shown in the Figure 1, 28 of 31 BLV infected cows (90.3%) > 8 years of age did not have lymphocytosis. These older cows showed a distinct pattern of Lym distribution from the younger cows, and were excluded from the analysis. The correlation coefficients (ρ) between age and logLym, age and log blood PVL/μl, and logLym and log blood PVL/μl were -0.25 ($p < 0.01$), -0.02 ($p = 0.78$), and 0.84 ($p < 0.01$), respectively. These data suggested significant correlations between logLym and age, and logLym and log blood PVL/μl in BLV infected cows.

Univariable GLM showed a significant linear relationship between logPVL/μl and logLym (slope = 0.84, SE = 0.03, $p < 0.01$), but not between logPVL/μl and age (slope = 0.11, SE = 0.06, $p = 0.10$). Table 2 shows the multivariable model result. Age, logLym and their interaction term was significant ($p < 0.01$), and the model was defined as Equation 1. The overdispersion parameter was 1921.5.

$$logPVL = 1.31 \times (Age) + 2.31 \times (logLym) - 0.14 \times (Age \times logLym) - 12.49 \quad \text{(Equation 1)}$$

Table 2. The final multivariable model result to predict BLV proviral load/μl

Variable	Estimate	Standard error	p-value
Intercept	−12.49	1.92	$p < 0.01$
Age	1.31	0.34	$p < 0.01$
LogLym	2.31	0.21	$p < 0.01$
LogLym:Age	−0.14	0.04	$p < 0.01$

1.3.4. Evaluation of the statistical model

The median and mean age of cows studied for verification were 4.48 and 4.65 years (2.5 and 97.5 percentiles: 2.15, 8.29), respectively. The median and mean Lym were 4,665.0 and 6,132.1 cells/μL of blood (2.5 and 97.5 percentiles: 2,283.8, 13,969.3), and median and mean PVL were 1,906.4 and 3,808.2 copies/μL of blood (2.5 and 97.5 percentiles: 1.5, 17,991.1), respectively. The dataset included 4 cows > 8 years of age; therefore, data from the remaining 88 blood samples were included in the verification.

The values of blood PVL/μl quantified by BLV-CoCoMo-qPCR and blood PVL/μl estimated from the statistical model were strongly correlated ($\rho = 0.87$, $p < 0.01$). The linear regression (equation 2), selecting the predicted blood PVL/μl using the equation 1 as an outcome variable, had the significant slope ($p < 0.01$), but it was smaller than 1 (slope = 0.74, SE = 0.05). This suggested that predicted blood PVL/μl tends to be overestimated when actual (measured) blood PVL/μl is low, whereas it is underestimated when actual blood PVL/μl is high (Figure 2).

$$Predicted\ blood\ PVL = 0.74 \times (Actual\ blood\ PVL) + 1686 \quad \text{(Equation 2)}$$

Figure 3 shows the relationship between logPVL/cell and log of white blood cell count/μl, they were strongly correlated ($\rho = 0.73$, $p < 0.01$), and PVL/cell was close to one among the cows with higher white blood cell count (Figure 3). Moreover, logPVL/cell was positively correlated with age as well ($\rho = 0.14$, $p = 0.04$, not shown in figures).

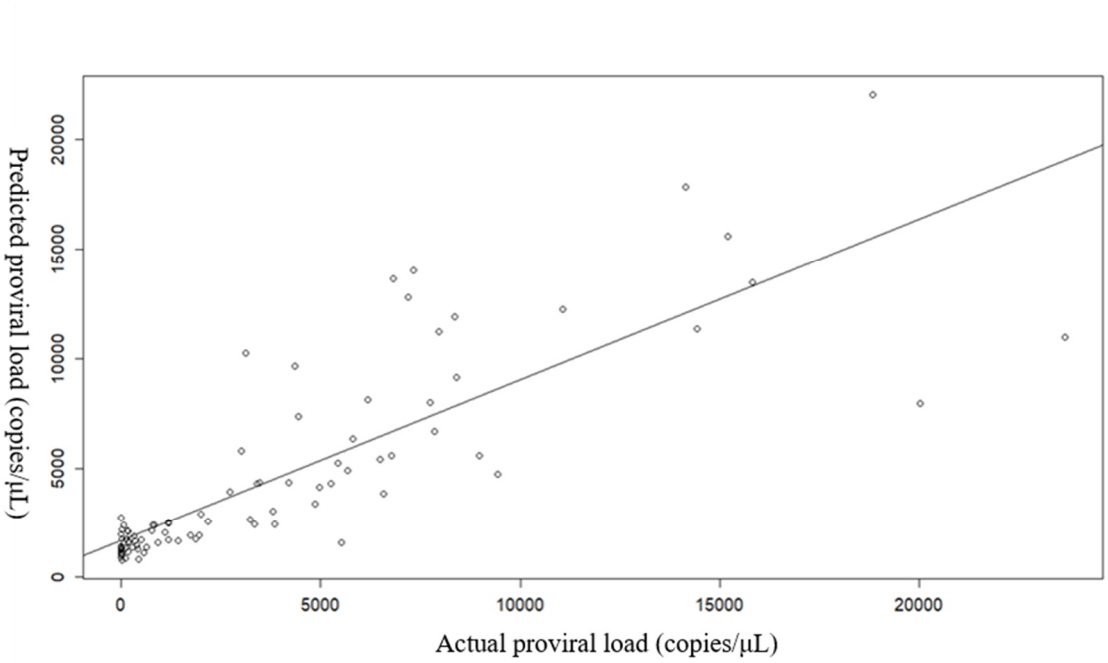

Figure 2. Scatter plot showing the relationship between predicted and actually measured proviral loads with a regression line

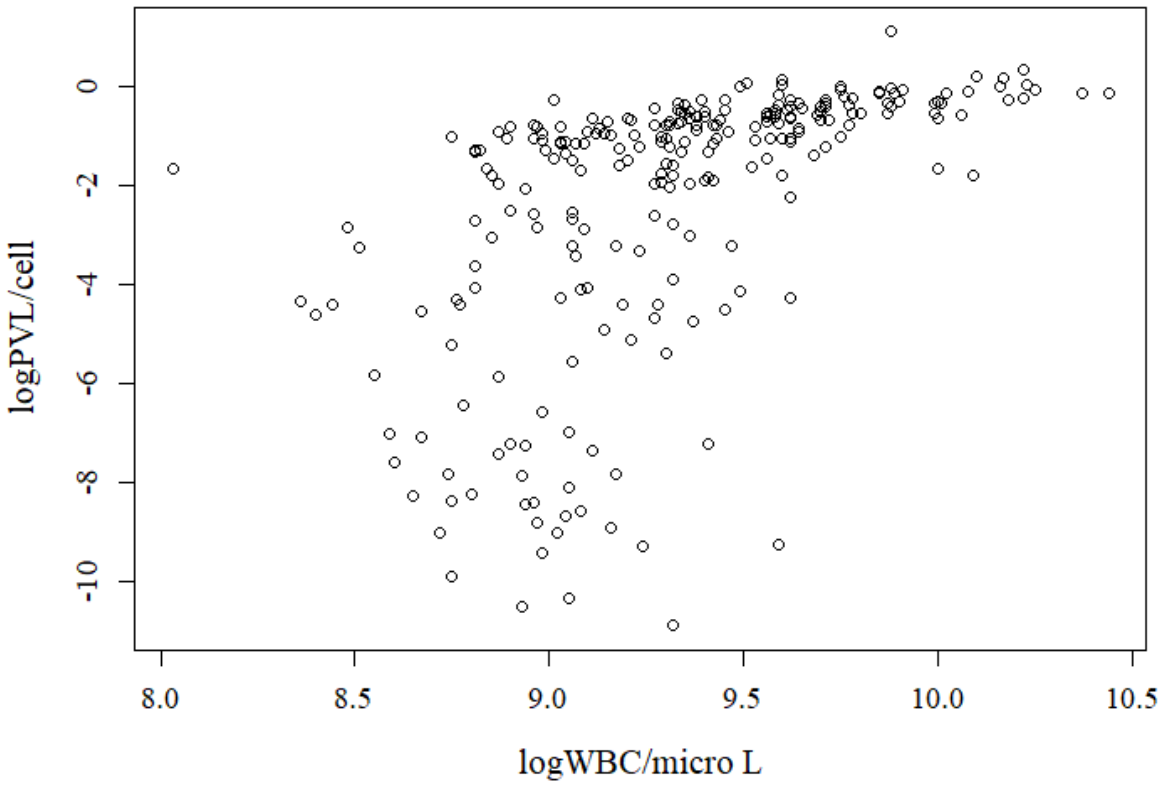

Figure 3. Scatter plot showing the relationship between logPVL/cell and log of white blood cell count/μl

1.4. Discussion

The measurement of PVL is not widely used in Japan, primarily due to the cost and labor involved to perform real-time PCR. This study demonstrated for the first time that a statistical model could be used as a substitute for quantitative real-time PCR.

This statistical model to estimate PVL consisted of two explanatory variables: age and logLym, and their interaction term. A positive relationship between PVL and Lym in BLV infected cows was previously reported [76]. However, a significant association between PVL and age has not been reported in previous studies [41, 63, 76]. Similarly, in my present study, I found no significant association between PVL/μl and age in the univariable analysis. There are two possible reasons why the statistical model required age. First, as shown in the correlation test result, as well as in the GLM for BLV non-infected cows, Lym decreases as a cow gets older. This aging effect has been reported in the reduction of both Lym [53] and white blood cell count [84] among BLV non-infected cows in the other studies. In my statistical model, the interaction term explains about this relationship. The second reason is the progression of lymphocytosis over age among BLV infected cows. BLV infected cows undergo a protracted AL stage with a low proviral load that continues for between 1 and 8 years [42], and 30% of BLV infected cows deteriorate into the PL stage [20, 42]. Moreover, the European Community's Leukosis Key or Bendixen's key is comprised of peripheral blood lymphocyte counts and age [1, 74]. Therefore, the positive relationship between age and PVL/μl in the multivariable model was considered biologically plausible.

Previous research reported that perinatal infection with BLV could quickly led to high PVL per cell [28]. Although Lym is higher in younger age as my study showed, the probability of developing lymphocytosis is limited in such age group; probability of transmission of infection is also determined by the number of infected Lym circulating [28, 51]. My study used blood PVL/μl as an outcome variable, and young stocks were included in the analysis. Therefore, my model is applicable to young animals as well. Moreover, particularly high PVL/cell among

young stocks were not observed in my study, and contribution of perinatal infection to the disease transmission in an endemic herd may not always be significant.

To demonstrate predictability of my model, I evaluated the model by comparing predicted and actually quantified PVL/μl values using data from two additional farms not included in the statistical modeling. However, as the slope of regression showed, my model may overestimate PVL/μl when actual PVL/μl is low, while underestimate when actual PVL/μl is high. Additional correlation tests showed that the low provirus productivity of the infected white blood cells among some proportion of non-lymphocytotic cows, particularly younger cows, caused this problem. However, as field veterinarians can send blood samples of potential highly efficient transmitters suggested by my statistical model to the laboratories for confirmation using quantitative PCR, this overestimation/underestimation problem would not discourage the field application.

Regarding the representativeness of the 10 commercial dairy farms used in this study, the mean number of dairy farms in the Nemuro region is 135.3 cows [35], and studied farms were considered to be representative for the region. The farms used in this study were not randomly sampled, and selection bias due to the influence of established strong relationship between the veterinarians and the farmers on the disease control may have existed. In the BLV-infected farms without good relationship with a veterinarian, BLV control may be delayed, and the proportion of lymphocytotic cows may be higher than the farms controlling BLV infections. The model estimated using data from such farms may show better predictability, as information from lymphocytotic cows would be weighed heavier. Although verification was conducted with two additional farms in my study, further studies may be needed for more robust validation.

In my dataset, 28 of 32 BLV-infected cows > 8 years of age (87.5%) did not develop lymphocytosis. In general, cows of all ages are susceptible to BLV; typically, infected cows > 3 years of age develop tumors (lymphosarcomas) [109]. Recently, genetic resistance to BLV infection was reported and bovine MHC class II BoLA-DRB3.2 allelic diversity was found to

be relevant to the progression of BLV infection [63, 71, 80, 105]. Therefore, BLV infected aged cows in this study that did not develop lymphocytosis might carry the resistance gene. In terms of the sensitivity of the exclusion of these older cows from statistical modelling, the mean calving number of cows that have been removed from farms was 4 [37], and my model based on the cows younger than 8 years old can be generally applicable to Japanese dairy population.

In conclusion, my statistical model for blood PVL/μl estimation based on Lym and age of BLV-infected cows, accompanied by quantitative PCR for the BLV-suspected cows, can be a cost-effective useful tool in prioritizing high risk cows to be removed from dairy herds to facilitate within-herd BLV control. I did not determine whether this statistical model could be applicable for use in BLV infected beef cattle and I did not consider the role of genetic resistance to BLV infection. I will need to examine these issues in the future.

1.5. Summary

Bovine leukemia virus (BLV) is widely prevalent in Japanese dairy farms. To control BLV infections in Japan, segregating or managing cows according to their proviral load (PVL) is a rational strategy. This study was conducted to establish a quantitative procedure for estimating blood PVL/μl using a statistical model to offer a cost-effective alternative to the conventional quantitative real-time PCR method. In total, 250 Holstein cows infected with BLV were identified from 10 commercial dairy farms. Information on age was collected, and blood samples were tested for blood white blood cell and lymphocyte counts and PVL using PCR. Generalized linear models with quasi-Poisson errors were used to estimate PVL selecting age, logarithm of lymphocyte count, and their interaction term as explanatory variables. To verify the model, blood samples of 92 BLV infected Holstein cows from two other commercial dairy farms were tested and measured PVL values were compared with estimated PVL values by the model. The logPVL/μl was modelled by positive associations with log lymphocyte count and age, and a negative association with the interaction term. In the verification, measured PVL values had a strong correlation with estimated PVL values (Spearman's ρ = 0.87). In conclusion, my model provides a cost-effective and more rapid alternative to the conventional method to facilitate test and segregation or management of BLV suspected cows.

Chapter 2. Loss of carcass weight in Japanese dairy cows due to infection with bovine leukemia virus

2.1. Introduction

In Chapter 1, I proposed the prediction model to estimate BLV PVL necessary for preventing BLV infection from based on age and blood lymphocyte count. As mentioned in the General introduction, Hokkaido has implemented eradication of infection of BLV. However, the number of cases of EBL increases every year and infection of BLV is considered to be spreading. In order to reduce prevalence of BLV in Hokkaido, it is necessary to estimate the economic loss due to BLV infection.

Several studies have described three types of significant economic losses from this disease. First, BLV infected cows survive shorter lifespans than non-infected cows [70]. Second, the milk production declines due to BLV infection both at the herd [18], and animal levels [73], though some studies failed to detect a significant difference in milk production between BLV-infected and non-infected animals [43, 94, 102]. Third, the carcasses of BLV-infected dairy cows are condemned at higher rates than those of non-infected animals, due to the presence of malignant lymphoma [5, 108]. Carcasses of dairy cows are an important source of beef [81], and in Japan, it accounted for 10.8% of the total amount of beef sold in 2017 [56]. Therefore, the increase in BLV prevalence in Japan has affected the efficacy of beef production in Japan.

In addition to these three types of economic losses, I hypothesized that another economic loss, reduction of carcass weight, may result from infection with BLV, given the degraded immunity of infected cows [20]. While the number of EBL cattle condemned at meat inspection in slaughterhouses has been increasing in Japan due to the increase in the prevalence of BLV [90], carcasses of BLV infected cows at the subclinical stage continue to be sold for consumption. To my knowledge, no previous study has quantified the economic loss associated with the reduced carcass weight of BLV-infected dairy cows.

Mediation analysis evaluates the relative magnitude of different pathways and mechanisms by which exposure may affect an outcome [104]. The objectives of the present study were to use mediation analysis to infer the causal relationship between infection with BLV, pathology

potentially caused by BLV-induced immunodeficiency, and carcass weight reduction; and to estimate the loss of carcass weight in the dairy industry in Hokkaido due to the BLV infection

2.2. Materials and methods

2.2.1. Study design

A prospective cohort study was used, selecting an exposed group as BLV-infected cows and a non-exposed group as BLV non-infected cows, to measure the effect of BLV infection on carcass weight. To infer the causal relationship between BLV infection and carcass weight loss in more detail, the role of BLV-induced immunodeficiency as a mediator was investigated using the number of visceral abnormal findings during post-mortem examination (AFPE).

2.2.2. Study areas and farm and cow selection

This study involved 12 BLV-infected commercial dairy farms in the Nemuro and Kushiro regions of Hokkaido, Japan. The BLV infection status of cows at these farms has been monitored by routine testing performed one or two times per year. The number of cows raised at these farms ranged between 57 and 435, with mean and median numbers of 163.2 and 125 cows, respectively. Within-farm prevalence of BLV infection ranged between 6.9% and 55.2%, with mean and median prevalences of 26.0% and 24.2%, respectively. Cows of known BLV status that were slaughtered within a week after removal from the farms between April 2015 and March 2018 were studied. The culled cows included in the present study were 2 years of age or older, and had previously given birth. BLV-infected cows in this study were defined as those cows that were diagnosed as being infected in the two most-recent consecutive blood tests (explained below) preceding slaughtering; non-infected cows were defined as those cows that were diagnosed as non-infected by the same tests.

2.2.3. Quantification of BLV PVL

Genomic DNA was isolated from whole blood samples using the Wizard Genomic DNA Purification Kit (Promega, Tokyo, Japan). The proviral load (PVL) was measured using either one of two real-time quantitative PCR assays, namely BLV-CoCoMo-qPCR (Riken Genesis,

Tokyo, Japan) or Cycleave BLV qPCR (Takara, Shiga, Japan).

BLV-CoCoMo-qPCR, which was used to ascertain BLV infection status and to gauge the PVL, was performed as described in my previous study [68]. The quantification of BLV PVL was performed at the Agricultural Research Department of Hokkaido Research Organization Animal Research Center.

The Cycleave BLV qPCR (Takara, Shiga, Japan) amplifies the tax region of BLV, and PVL assess by this test was expressed as the number of proviral copies per 50 ng of DNA. The quantification of BLV PVL was performed at the Hokkaido Higashi Agriculture Mutual Aid Association clinical laboratory or at the Research Institute for Animal Science in Biochemistry and Toxicology.

The field veterinarians decided on the use of quantitative PCR assays employing either BLV-CoCoMo-qPCR or the Cycleave BLV qPCR, depending on access to the corresponding laboratories.

2.2.4. Classification of the level of BLV PVL

The BLV-infected cows were classified into two groups according to the PVL: low PVL (L-PVL) and high PVL (H-PVL). The cut-off thresholds used to classify the PVL levels for both PCR methods were determined using receiver operating characteristic curve analysis [91] and employing the European Community (EC)'s key for lymphocytic status, which is based on the absolute lymphocyte count and age of a cow [53]. Specifically, blood samples were collected from 69 apparently healthy but BLV-infected Holstein cows from a commercial dairy farm in the Kushiro region, and lymphocyte count (Lym), and BLV PVL quantifications were performed at the Agricultural Research Department of Hokkaido Research Organization Animal Research Center using both BLV-CoCoMo-qPCR and Cycleave BLV qPCR. Using the age and Lym information, these cows were classified as lymphocytic or not based on the EC's key. The optimal cut-off thresholds of PVL measurement to classify infected cows as H-PVL

or L-PVL were calculated as 56,767 copies/10^5 cells (for BLV-CoCoMo-qPCR; area under the curve (AUC) = 0.96) and 2,464.8 copies/50 ng DNA (for Cycleave BLV qPCR; AUC = 0.97), using the pROC software package [82], in the context of classification based on the EC's key.

Notably, the robustness of statistical inference based on classification using either of the two PVL quantification methods was supported by the high Spearman's rank correlation (0.97, $p < 0.01$) between the BLV-CoCoMo-qPCR and Cycleave BLV qPCR results. Furthermore, the EC's key is based on the absolute lymphocyte count [53], while BLV-CoCoMo-qPCR and the Cycleave BLV qPCR BLV assess PVL per unit of white blood cell. My previous report showed that, as the white blood cell count of a BLV-infected cow increases, PVL also approaches a maximum value of one copy per white blood cell [68]. This observation meant that results obtained using my classification method, determined based on the EC's key, were plausible.

L-PVL and H-PVL cows were defined as those cows whose PVLs were below and above (respectively) the cut-off thresholds in the two most-recent consecutive tests before slaughtering. Cows with contradictory results for classification as either L-PVL or H-PVL in the two most-recent consecutive tests were excluded from the present study.

2.2.5. Data collection and management

Herd-level information (such as a total number of cows and the number of culled cows) and animal-level information (such as age, parity, and BLV test results of culled cows and the postpartum days at slaughtering) were collected by the field veterinarians in charge of the study farms through interviews with the farm owners and checking of the farm records.

Cow ages were categorized as 2, 3, 4, or 5 and over years old. None of the 2-year-old culled cows were infected with BLV; animals in this age range were excluded from all the statistical analyses. The median calving interval for dairy cows in Hokkaido was 407 days [47], and cows culled at more than 1,000 days from the last delivery may be outliers. Therefore, culled cows with periods of greater than 1000 days since calving were excluded from the analysis. The

lactation stages were assigned to 4 categories based on time since delivery: early lactation, 0 to 49 postpartum days; peak lactation, 50 to 109 days; mid-lactation, 110 to 209 days; and late lactation, 210 days or longer.

The destinations of culled cows were traced using the database of the Japan National Livestock Breeding Center. All culled cows used for this study were confirmed not to have exhibited any clinical signs suggestive of EBL from the clinical records. The carcass weight data of culled cows were collected from meat processing wholesalers after obtaining informed consent from the farm owners.

The post-mortem examination records of the culled dairy cows were collected from the Department of Health and Welfare, the Hokkaido Prefectural Government, after obtaining informed consent from the farm owners. These records included abnormal findings related to the uterus: the postpartum uterus and pregnant uterus; however, these items were not pathological findings and were excluded from this study. Animals whose whole carcasses were condemned due to sepsis, edema, jaundice, and uremia also were excluded, because these condemnations were due to a systemic symptom, preventing determination of the primary lesion.

For the purposes of analysis, the number of AFPE was counted for all the culled dairy cows studied. In the present study, no culled cow had its whole carcass condemned due to post-mortem examination results. All the data were digitized and handled using commercially available spreadsheet software (Excel 2013; Microsoft Corp., Redmond, WA, USA).

2.2.6. Statistical analysis

Descriptive statistics were performed by calculating the mean, median, interquartile range, and 2.5 and 97.5 percentiles for the variables collected. The data of carcass weight were right-skewed, and the logarithms of these data exhibited normality as assessed by the Shapiro-Wilk test.

For univariable analyses, generalized linear mixed-effects models (GLMMs) with log-link gamma errors were performed, selecting carcass weight as an outcome variable, identification of herds as a random effect, and BLV infection status, the number of AFPE, age, and lactation stage as explanatory variables. In addition to the analyses, the relationship between AFPE and carcass weight was analyzed using a generalized linear model (GLM) with log-link gamma errors.

For the multivariable causal inference of the association between BLV infection status and carcass weight loss; and for measurements of direct, indirect, and total carcass weight losses, three models were used: (1) prediction of carcass weight loss by BLV infection status (Model 1), (2) prediction by BLV infection status and AFPE in a linear model (Model 2), and (3) prediction by BLV infection status mediated by AFPE (Model 3). Multi-collinearity between variables was diagnosed by the generalized variance inflation factor (GVIF): when GVIF is 2 or higher [19], the variables are considered to have multi-collinearity.

For Model 1 (Equation 3, Figure 4a), a GLMM with log-link gamma errors was performed.

$$E(Y_i) = \nu + \varphi_1 B_i + \varphi_2 C_i + H_j + e_{ij} \quad \text{(Equation 3)}$$

where Y_i is the carcass weight for ith culled cow (i = 1, 2,…, 83), v is overall mean, B_i is BLV infection status (non-infected, L-PVL and H-PVL), C_i represents indicator variables for the known confounders (age and lactation stage) related with B_i and Y_i, φ_1 is the total effect of B_i, and φ_2 is a regression coefficient for C_i. H_j is herd identification as a random effect (j = 1, 2,…, 12); e_{ij} expresses an error term for the carcass weight for ith culled cow in jth herd.

Model 2 additionally included AFPE as an explanatory variable (Equation 4).

$$E(Y_i) = \nu + \varphi_1 B_i + \theta_2 A_i + \varphi_2 C_i + H_j + e_{ij} \quad \text{(Equation 4)}$$

where A_i is the number of AFPE. The degree of interaction of the AFPE on the effect of BLV infection status on the reduction of carcass body weight, as an indirect effect, was measured using the difference method of mediation analysis [104]. The change of the estimates for BLV infection status between Model 1 and Model 2 was calculated by dividing the differences in the estimates for L-PVL and H-PVL by the respective estimates in Model 1. Since the GLMMs applied a log function, the carcass weight loss was calculated by exponential transformation. This statistical analysis was applied using R package lem4 version 1.1-23 [8].

After checking the indirect effect of the AFPE on the carcass body weight, more accurate measurements of the direct effect by the BLV status and of the indirect effect by the AFPE on carcass body weight were conducted using Bayesian mediation analysis (Model 3, Figure 4b). Model 3 consisted of two equations: the Bayesian GLMM with gamma errors (Equation 5), which includes the mediator (M_i); and the M_i, Bayesian GLMM with Poisson errors (Equation 6).

$$E(Y_i) = \mu + \theta_1 B_i + \theta_2 M_i + \theta_3 C'_i + H_j + e_{ij} \quad \text{(Equation 5)}$$

$$E(M_i) = \lambda + \sigma_1 B_i + \sigma_2 C''_i + H_j + e_{ij} \quad \text{(Equation 6)}$$

where M_i is the number of AFPE for *i*th culled cow; μ and λ are the overall means; C'_i represents confounders (age and lactation stage) related with BLV infection status and carcass weight for the culled cow; C''_i represents a confounder, age, related with BLV infection status and the AFPE (it is unlikely that a particular lactation stage increases the AFPE); and θ and σ are the regression coefficients (Figure 4b). θ_1 is the direct effect of BLV infection status, and the product of θ_2 and σ_1 is the indirect effect.

Model 3 was constructed via Markov Chain Monte Carlo (MCMC) using the R package brms 2.12.0 [9], rstan 2.19.3 [25], and sjstats 0.17.9 [49]. Uniform distribution was selected for the prior distributions; the sample size and length of burn-in were decided based on visual inspections of trace plots. Samples were derived by the MCMC algorithm, using 4 chains with

10,000 iterations (1000 warm-up samples for each chain) and 36,000 samples. Unstandardized effect estimates and 95% credible intervals (CreI) were reported. All statistical analyses were performed using R version 3.6.0 and R studio 1.2.5042 [96, 97].

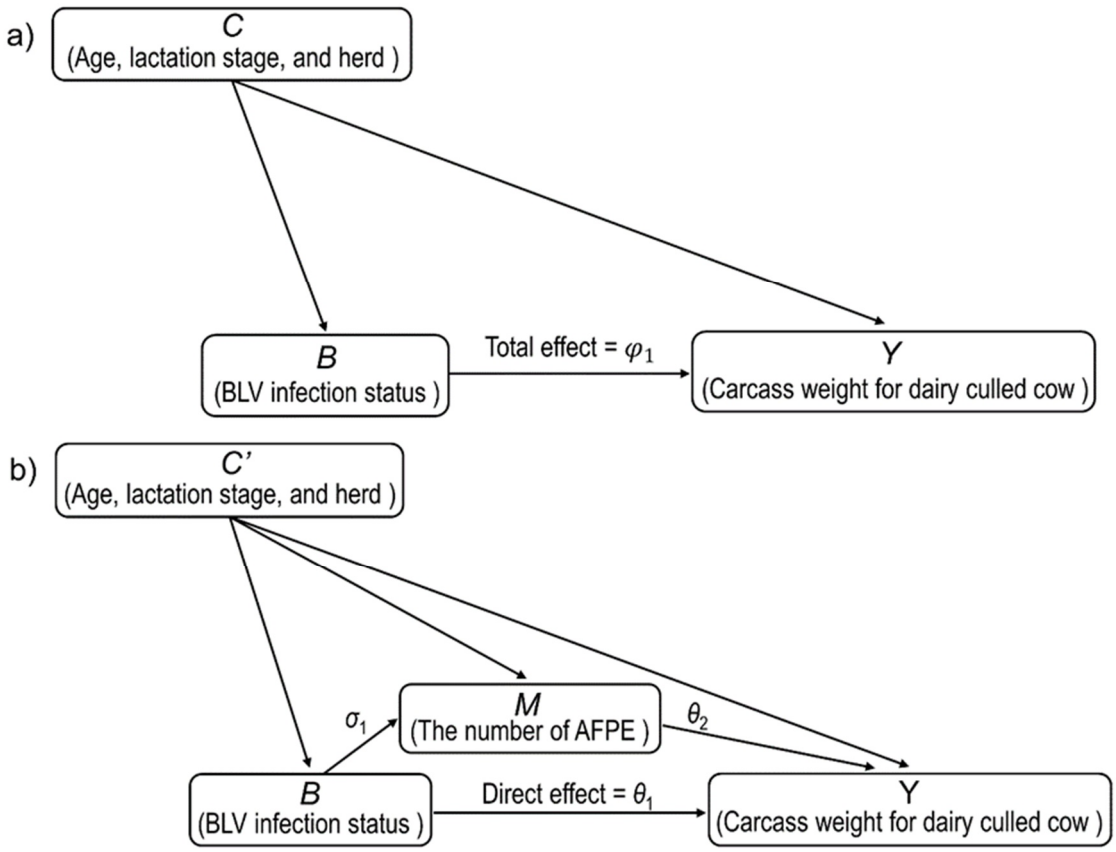

Figure 4. Causal webs for carcass weight loss. Panel (a) shows Model 1, which describes the total effect of BLV infection status on carcass weight, with potential confounders. Panel (b) shows Model 3, the mediation analysis model. θ_1 indicates the strength of the direct effect of *B* (BLV infection) on *Y* (carcass weight), and θ_2 and σ_1 indicate the strengths of the effects of *B* on *M* (AFPE), and *M* on *Y*, respectively. The indirect effect is calculated in panels (a) and (b) as: $\varphi_1 - \theta_1 = \sigma_1 \times \theta_2$. The parameters φ_1, φ_3, θ_1, θ_2, θ_3, σ_1, and σ_3 are the regression coefficients relating to Equations 3 to 6 in the main text.

2.2.7. Estimation of economic loss

The annual economic loss of carcass weight due to BLV infection in Hokkaido Prefecture in 2017 was calculated by multiplying the total carcass weight loss posterior distributions for H-PVL and L-PVL cows (estimated using Bayesian mediation analysis) with the estimated number of BLV-infected cows culled in a year, and by the unit price of one kilogram of meat for a culled dairy cow, using a Monte Carlo simulation. The information on the number of culled dairy cows slaughtered in Hokkaido in 2017 was collected from the Hokkaido Prefectural Government Department of Health and Welfare. The proportion of BLV-infected cows among culled dairy cows in Hokkaido (11.5%) was taken from the previous prevalence report [64]. Using the beta distributions modeled for the proportions of L-PVL and H-PVL cows among BLV-infected cows in the research data, the numbers of L-PVL and H-PVL dairy cows slaughtered in Hokkaido in 2017 were estimated. The unit price per carcass for culled dairy cows was 560 yen/ kg (which is 5.3 US dollars based on the 2020 September 6 exchange rate), based on a survey published by the Ministry of Agriculture, Forestry and Fisheries [58].

2.3. Results

2.3.1. Descriptive Statistics

Out of the 330 heads of Holstein culled cows in 12 farms, 222 culled cows were studied after the exclusion mentioned above. According to the BLV tests, 11 cows were H-PVL, 32 were L-PVL, and 179 were not infected with BLV

Figure 5 shows the distribution of carcass weight, and the mean, median, and interquartile range were 299.2, 284.0, and 113.5 kg, respectively. The descriptive statistics for carcass weight, age, the number of AFPE, and lactation phase by BLV infection status are shown in Table 3. The mean AFPE showed an increasing trend over the progression of BLV infection status. The most frequent condemned organs among BLV-infected cows were liver, heart, and ruminant stomach: forestomach and abomasum; the proportions, were 67.4% (95% confidence interval (CI): 51.3%, 80.5%), 27.9% (95% CI: 15.8%, 43.9%), and 16.3% (95% CI: 7.3%, 31.3%), respectively.

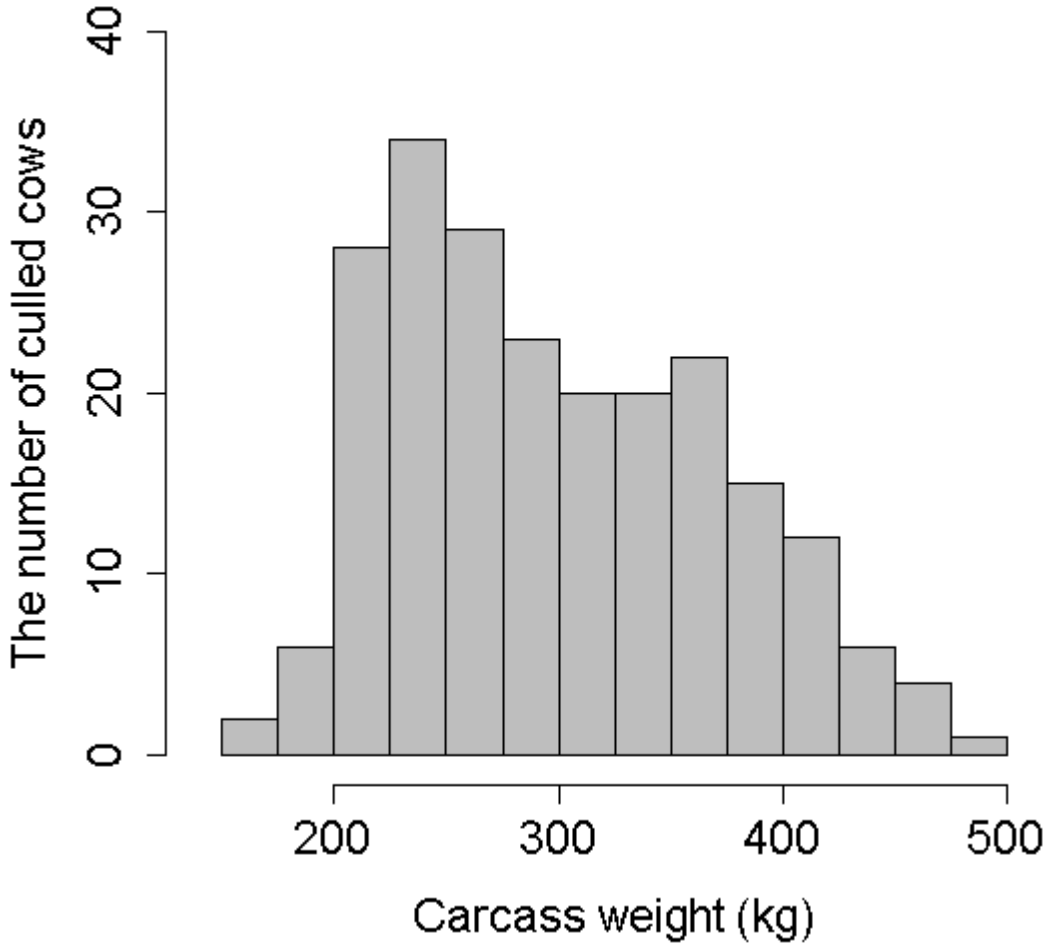

Figure 5. A histogram of the carcass weight of culled dairy cows (n=222).

Table 3. Descriptive statistics for the BLV infection status of culled dairy cows from 12 farms in Hokkaido, Japan, between April 2015 and March 2018.

Variable	Median (2.5-97.5th percentiles)			The number of cows (%)			
	Carcass weight	Age	AFPE[a]	Early lactation	Peak lactation	Mid-lactation	Late lactation
Non-infected	285 (196.8-450.3)	5.2 (3.1-11.9)	1 (0-5)	8 (4.5)	19 (10.6)	38 (21.2)	114 (63.7)
Low-proviral-load	290 (202.0-419.2)	6.6 (3.4-10.8)	1.5 (0-6)	3 (9.4)	3 (9.4)	4 (12.5)	22 (68.7)
High-proviral-load	264 (192.0-391.1)	5.6 (3.6-8.2)	3 (0-5.8)	1 (9.1)	1 (9.1)	0 (0)	9 (81.8)

AFPE[a]: abnormal finding of the postmortem examination

2.3.2. Univariable analysis

Table 4 shows the univariable analysis results for the factors associated with the carcass body weight of culled dairy cows, among categorical variables. Age and lactation phase were associated with carcass weight, whereas BLV infection status was not.

The number of AFPE was negatively associated with carcass weight (slope = -0.047, standard error (se) = 0.01, $p < 0.01$).

Table 4. Univariable analysis results for the factors associated with carcass body weight of culled dairy cows among categorical variables.

Variable	Median carcass weight	2.5-97.5th percentiles	Number of cows	*p*-value
BLV infection status				
Non-infected	285.0	238.5 - 355.5	179	Reference
Low-proviral-load	290.0	261.8 - 354.0	32	0.89
High-proviral-load	264.0	239.1 - 308.7	11	0.30
Age				
3 to 4	242.3	217.8 - 311.3	40	Reference
4 to 5	280.0	228.8 - 356.0	46	0.03
Over 5 years	299.0	256.0 - 357.5	136	<0.01
Lactation stage				
Early	239.6	226.8 - 268.0	12	Reference
Peak	239.0	223.5 - 263.0	22	0.41
Middle	258.0	229.0 - 300.5	43	0.88
Late	322.0	266.0 - 369.0	145	<0.01

2.3.3. Measurement of the degree of interaction of the AFPE on the effect of the BLV infection status on the reduction of carcass body weight

GVIFs of Model 1 and Model 2 approached 1, and multi-collinearity was not observed. The result of Model 1 (Table 5) showed that a mean carcass weight for H-PVL culled cows was significantly lighter, by 30.2 kg, than that of non-infected cows (estimate = -0.14, $p = 0.03$) (total effect). The mean carcass weight of late-lactation culled cows was significantly heavier, by 47.5 kg, than that of the early-lactation culled cows (estimate = 0.19, $p < 0.01$). The mean carcass weight for culled cows aged 5 years or older was significantly heavier, by 35.0 kg, than that of culled cows aged 3 and 4 years old (estimate = 0.14, $p < 0.01$).

The results of Model 2 (Table 6) indicated that when AFPE increased by 1, carcass weight decreased by 8.55 kg (estimate = -0.04, $p < 0.01$). The difference in mean carcass weights for culled early- and late-lactation stage cows was slightly smaller (42.0 kg, estimate = 0.16, $p < 0.01$) than that seen in Model 1 (estimate = 0.19, Table 3). BLV infection status was not significantly associated with carcass weight in this model. Age remained a significant factor for carcass weight.

The change of the estimates for H-PVL cows between Models 1 and 2 was 42.9% (from -0.14 to -0.08), and that for L-PVL cows was 0% (-0.06 in both models), suggesting the existence of indirect effect of H-PVL, through AFPE, on carcass weight loss.

Table 5. Statistical relationship between carcass weight of culled dairy cows and BLV infection status with confounders (Model 1)

Variable	Estimate	Standard error	*P*-value
Fixed effects			
Intercept	5.44	0.08	<**0.01**
BLV infection status			
Non-infected	Reference		
Low-proviral-load	-0.06	0.04	0.12
High-proviral-load	-0.14	0.06	**0.03**
Age			
3 to 4	Reference		
4 to 5	0.06	0.04	0.10
Over 5 years	0.14	0.03	<**0.01**
Lactation stage			
Early	Reference		
Peak	-0.07	0.07	0.27
Middle	0.00	0.06	0.97
Late	0.19	0.06	<**0.01**
Random effect	Variance	Standard deviation	ICC[a]%
Herd	0.01	0.09	14.9

ICC[a]: Intraclass Correlation Coefficient

Table 6. Statistical relationships between carcass weight and BLV infection status with confounders, adjusted by the number of AFPE (Model 2)

Variable	Estimate	Standard error	*P*-value
Fixed effect			
Intercept	5.5	0.07	<**0.01**
BLV infection status			
Non-infected	Reference		
Low-proviral-load	-0.06	0.04	0.17
High-proviral-load	-0.08	0.06	0.17
Age			
3 to 4	Reference		
4 to 5	0.09	0.04	**0.02**
Over 5 years	0.16	0.03	<**0.01**
Lactation stage			
Early	Reference		
Peak	-0.07	0.06	0.23
Middle	-0.01	0.06	0.83
Late	0.16	0.05	<**0.01**
The number of AFPE[a]	-0.04	0.01	<**0.01**
Random effect	Variance	Standard deviation	ICC[b]%
Herd	0.01	0.08	16.15

AFPE[a]: abnormal finding of the postmortem examination

ICC[b]: Intraclass Correlation Coefficient

2.3.4. Mediation analysis for the estimation of precise effects

Table 7 shows the results of the mediation analysis. The distributions of direct effects of BLV infection on carcass weight included areas with different signs for both H-PVL and L-PVL, though the negative medians suggested some effects in reducing carcass weight. The AFPE significantly reduced carcass weight (median = −0.04, 95% CreI: −0.06 to −0.03). BLV H-PVL significantly increased AFPE (0.48, 95% CreI: 0.06 to 0.88).

The distributions of indirect effects of BLV infection on carcass weight through AFPE, calculated as the product of the effect of AFPE on carcass weight and the effect of BLV infection on AFPE, showed that the indirect effect was significant for H-PVL culled cows (credible interval did not include the areas with different signs), but was not significant for L-PVL culled cows (Table 8). Moreover, the total effects of BLV infection on carcass weight were not significant (Table 8). The proportion of the effect mediated by AFPE was 21.56% for H-PVL culled cows, a value that was much larger than the 8.74% obtained for L-PVL culled cows. Figure 6 shows the posterior distributions of the direct and indirect effects of BLV L-PVL and H-PVL on carcass weight.

Table 7. Bayesian mediation analysis results showing estimates (in logarithmic units)

Variables	Median	Standard Error	95% credible intervals
Carcass weight intercept	5.52	0.07	5.38, 5.67
AFPE [a] intercept	0.14	0.18	-0.22, 0.48
Estimates for carcass weight			
BLV status: non-infected	Reference		
BLV status: low-proviral-load	-0.05	0.04	-0.13, 0.03
BLV status: high-proviral-load	-0.08	0.06	-0.21, 0.04
Age: 3 years old	Reference		
Age: 4 to 5 years old	0.09	0.04	-0.13. 0.03
Age: over 5 years old	0.16	0.03	0.10, 0.23
Lactation stage: Early	Reference		
Lactation stage: Peak	-0.07	0.06	-0.20, 0.05
Lactation stage: Middle	-0.01	0.06	-0.20, 0.05
Lactation stage: Late	0.16	0.05	0.05, 0.26
AFPE	-0.04	0.01	-0.06, -0.03
Estimates for AFPE			
BLV status: non-infected	Reference		
BLV status: low-proviral-load	0.10	0.16	-0.21, 0.41
BLV status: high-proviral-load	0.48	0.21	0.06, 0.88
Age: 3 years old	Reference		
Age: 4 to 5 years old	0.38	0.19	0.02, 0.76
Age: over 5 years old	0.36	0.16	0.06, 0.69
Standard deviation for carcass weight	0.14	0.04	0.08, 0.23
Standard deviation for AFPE	0.28	0.12	

AFPE[a]: abnormal finding of the postmortem examination

Table 8. Direct, indirect and total effects of BLV infection on carcass weight losses (95% credible intervals)

BLV infection status	Direct	Indirect	Total
Low- viremia	-0.05	-0.00	-0.06
	(-0.13 to 0.03)	(-0.02 to 0.01)	(-0.14 to 0.02)
High- viremia	-0.08	-0.02	-0.10
	(-0.21 to 0.04)	(-0.04 to -0.00)	(-0.23 to 0.02)

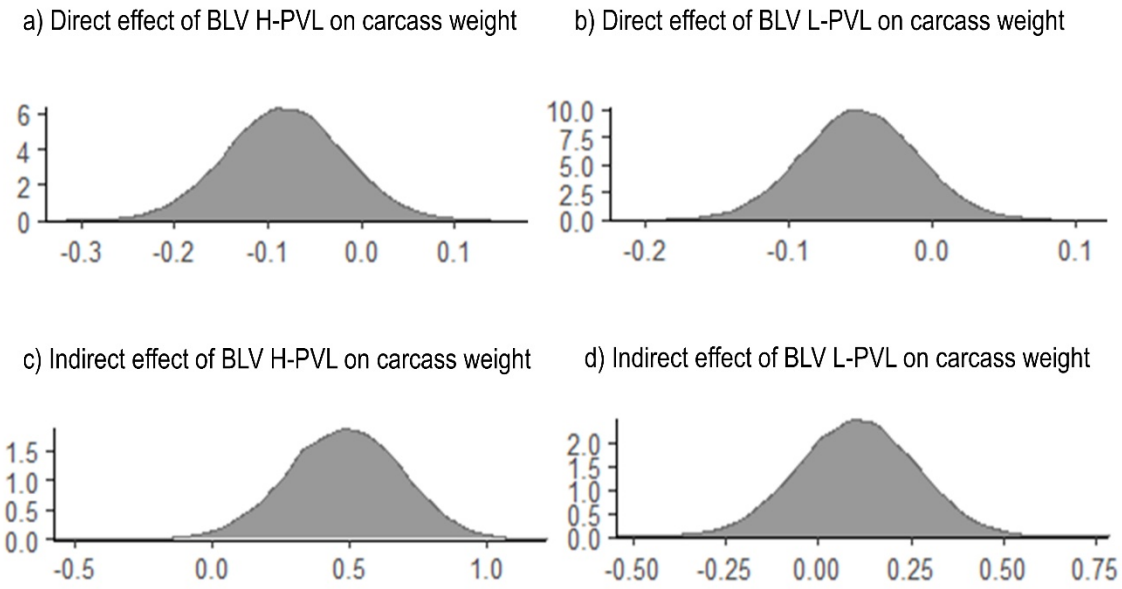

Figure 6. Posterior distributions of precise direct and indirect effects of BLV infection on carcass weight, as estimated by the mediation analysis. The x-axes show the value spaces, and the y-axes show the probability densities. Panels (a) and (b) show the direct effects of BLV H-PVL and L-PVL on carcass weight, respectively; and panels (c) and (d) show the respective indirect effects.

2.3.5. Estimation of the economic loss

The median annual direct loss of carcass weight due to BLV infection in Hokkaido Prefecture in 2017 was 118,166 (2.5 and 97.5 percentiles: 54,087, 180,789) kg, the annual economic loss was 66,172,680 (2.5 and 97.5 percentiles: 30,288,552, 101,241,952) yen, and 626,277 (2.5 and 97.5 percentiles: 286,656, 958,183) US Dollars.

2.4. Discussion

This study investigated the causal relationships among BLV infection status, the number of AFPE, and carcass weight of culled dairy cows (which contribute to the food chain), adjusted for age, lactation stage, and owner. In these tripartite relationships, I hypothesized that BLV infection caused carcass weight loss, mediated by an increased number of AFPE due to degraded immune status. My results suggested that mediation exists for H-PVL cows.

This work represents the first prospective cohort study investigating the influence of BLV infection on bovine carcass weight, using meat inspection records, to my knowledge. In other work, pre- and post-mortem inspection data have been utilized to ensure the safety of meat products and to plan control strategies for infectious diseases at the farm level [2, 3, 61, 99]. The present study is unique because this analysis further applied the inspection data for understanding multiple causations in economic loss due to BLV, using the information collected at farms.

Malignant lymphoma, which is the pathology most strongly associated with BLV infection, is one of the 4 most common reasons for cow carcass condemnations in the US [108], and it would be easy to estimate the resulting loss in Japan as well. However, that aspect was beyond the scope of my study. The annual economic loss due to carcass weight loss in Hokkaido Prefecture, which has not been determined previously, was not negligible (US $626 thousand); this value would be an important piece of information for farmers considering participation in voluntary BLV control programs.

The mediation analysis proved the significant effect of immunosuppression on carcass weight, as the credible interval of indirect effect on carcass weight caused by AFPE did not include zero (or was marginal) for H-PVL cows. BLV causes immune deregulation after the primary infection stage [31], and H-PVL cow becomes immunosuppressed [21]. I hypothesize that the increased number of AFPE among BLV-infected cows reflects immunosuppression due

to BLV infection. According to the literature, most of the macroscopic lesions caused by BLV infection are found in the digestive organs such as omasum, abomasum, intestine, liver, and spleen; clinical signs depend on the site of formation of macroscopic lesions, leading to symptoms such as digestive disturbances, inappetence, weight loss, and weakness [109]. My results also showed similar findings, suggesting that the damage of digestive organs caused by BLV infection affected the maintenance of carcass weight.

Carcass weight loss also may be a direct result of BLV infection. Although the credible intervals of direct effects of L-PVL and H-PVL cows included zero, the distributions suggested that a direct effect may exist (Figure 6). As bovine leukemia progresses, the numbers of both BLV-PVL copies per cell and lymphocyte increase [68]. The organs that incorporate large number of lymphocytes therefore harbor BLV at high copy number; for instance, in a previous study conducted in a slaughterhouse, lymph nodes and spleens from EBL cows had higher BLV PVL than those from asymptomatic BLV-infected cows [92]. Immunodeficiency arises as BLV infection develops to the PL stage [42], thus, H-PVL may induce carcass weight loss through malfunction of other organs than those that showed abnormal pathology.

The large calculated mediation resulted from carcass loss, which itself reflected increased AFPE caused by H-PVL; these losses confirm the importance of priority culling of H-PVL cows for controlling BLV infection [88]. In the dairy industry, the detection and culling of H-PVL cows are critical not only for BLV infection control but also for securing income from carcasses. Another strategy for preventing economic loss from BLV infection is to prevent the development of high BLV viremia in infected cows. For this strategy, two approaches can be explored. The first approach is the use of cattle bred to be tolerant to BLV. Specifically, bovine lymphocyte antigen class II haplotypes are associated with BLV PVL [63], and cattle breeds lacking these haplotypes may be BLV tolerant. The second approach is the treatment of BLV using immunoinhibitory molecules, such as Programmed cell death protein 1 (PD-1) and its ligand (PD-L1), that have been shown to be effective against cows with high levels of viremia

[89].

This study suffers from 2 limitations related to field data. First, this study employed two separate assays for quantification of BLV PVL. According to a previous report, significant unevenness in PVL quantification was observed among different laboratories [38]. However, a strong correlation between the two methods: (BLV-CoCoMo-qPCR and Cycleave BLV qPCR) was observed in my study, suggesting that differences in the testing methods may not have served as a significant limitation in the present work. Second, the BLV prevalence used in this study was based on 2009-2011 data; since then, the number of EBL notifications has tripled compared to a decade ago [59]. Therefore, the economic losses estimated in this study might be large underestimated.

In conclusion, this study revealed that infection with BLV decreased the carcass weight of culled dairy cows and resulted in significant economic loss in Hokkaido Prefecture. My results indicated that persistent high BLV PVL causes a reduction in carcass weight due to an increased number of AFPE. Priority culling of high-viremia cows is a reasonable strategy in terms of both BLV infection control and economics.

2.5. Summary

Bovine leukemia virus (BLV) infection is endemic in Japanese dairy farms. To promote the participation of farmers in BLV infection control in Japan, it is important to provide estimates of the economic losses caused by this infection. I hypothesized that decreased immune function due to BLV infection would increase visceral abnormalities, in turn reducing carcass weight. I employed mediation analysis to estimate the annual economic loss due to carcass weight reduction caused by BLV infection.

Culled Holstein cows from 12 commercial dairy farms in the Nemuro and Kushiro regions of Hokkaido, Japan, were traced; the analysis included culled cows whose BLV proviral loads were known through the routine monitoring scheme. Information on age and the last delivery day were collected at the farms. A non-infected culled cow was defined as a cow from which BLV provirus was not detected just before sale to a slaughterhouse. Low-proviral-load (L-PVL) and high-proviral-load (H-PVL) cows were defined as cows whose PVL titers were below and above 2,465 copies/50 ng DNA respectively, or below and above 56,765 copies/10^5 cells respectively, as assessed by two consecutive blood tests performed immediately before sale to a slaughterhouse. Post-mortem examination results for the culled cows were collected from a meat inspection center.

The hypothesis was tested by three models, using data from 222 culled dairy cows. Model 1, a generalized linear mixed-effects model, selected carcass weight as an outcome variable, BLV status and the potential confounders (lactation stage and age) as explanatory variables, and farm identification as a random effect. Model 2 additionally included the number of abnormal findings in the post-mortem examination (AFPE) as an explanatory variable. Model 3 applied a Bayesian generalized linear mixed model, which employed a mediator separately modeled for AFPE, to estimate the amount of direct, indirect, and total carcass weight loss with adjustment

for known confounding factors.

Compared to the mean carcass weight for the non-infected culled cows, the carcass weight for H-PVL-infected culled cows was significantly decreased, by 30.4 kg. For each increase of one in the number of AFPE, the mean carcass weight was decreased by 8.6 kg. Only the indirect effect of BLV H-PVL status on carcass weight loss through AFPE was significant, accounting for 21.6% of the total effect on carcass weight reduction. In 2017, 73,650 culled dairy cows were slaughtered in Hokkaido, and the economic loss due to carcass weight loss caused by BLV infection that year was estimated to be US $626 thousand. In summary, unlike L-PVL cows, H-PVL status was associated with carcass weight reduction, which appeared to be mediated by an increase in the number of visceral abnormalities.

Chapter 3. Economic loss of mastitis associated with bovine leukemia virus infection

3.1. Introduction

In Chapter 2, I proved that decreased immune function due to BLV H-PVL increased visceral abnormalities, which reduces the carcass weight. I employed a mediation analysis and estimated the annual economic loss of carcasses weight reduction due to BLV infection.

In 2013, the most common clinical disease in dairy cows was mastitis in the USA [66]. In the USA, the annual economic loss due to mastitis was estimated at between 1.7 and 2.0 billion US dollars [45], and that in Canada was 400 million Canadian dollars [10]. Nutrition, host resistance, environmental conditions, milking equipment, milking technique, and hygiene are known to be associated with risk of mastitis [46]. BLV infection induces abnormal immune function [20] that can lead to mastitis. To the best of our knowledge, no study has examined the relationship between BLV infection and mastitis in consideration of these mastitis risk factors.

The objectives of the present study were to infer the association between BLV infection status and occurrence of mastitis and estimate the annual economic loss due to mastitis caused by BLV infection in Hokkaido Prefecture, the main dairy production area of Japan.

3.2. Materials and methods

3.2.1. Study design and field investigation

A retrospective cohort study involving 9 commercial dairy farms with BLV-infected cows was conducted. These farms, located in the Nemuro and Kushiro regions of Hokkaido Prefecture, Japan, are routinely surveyed once or twice per year to determine the BLV infection status of cows. The study was conducted between April 2015 and March 2018. No cows within the 9 herds were diagnosed with EBL during the study period.

3.2.2. Quantification of BLV proviral load (PVL)

Genomic DNA was isolated from whole blood samples using a Wizard Genomic DNA Purification kit (Promega, Tokyo, Japan). PVL was measured using one of two different real-time quantitative PCR assays, namely the BLV-CoCoMo-qPCR (Riken Genesis, Tokyo, Japan) or Cycleave BLV qPCR (Takara, Shiga, Japan) assay. The field veterinarians in charge decided using either the BLV-CoCoMo-qPCR or Cycleave BLV qPCR assay. Good agreement between the results of these assays was described in my previous studies [68].

Quantification of BLV-PVL using the BLV-CoCoMo-qPCR assay was performed at the Agricultural Research Department of the Hokkaido Research Organization Animal Research Center, and that using the Cycleave BLV qPCR assay was performed at the Hokkaido Higashi Agriculture Mutual Aid Association clinical laboratory or the Research Institute for Animal Science in Biochemistry and Toxicology.

3.2.3. Classification of BLV PVL

BLV-infected cows were classified into two groups according to PVL: low PVL (L-PVL) and high PVL (H-PVL). According to my previous research, the L-PVL and H-PVL cut-off thresholds as determined using the BLV-CoCoMo-qPCR and Cycleave BLV qPCR BLV assays were 2,465 copies/50 ng DNA, and 56,765 copies/10^5 cells, respectively. L-PVL and H-PVL

cows were defined as those in which the PVL was below and above the indicated cut-off thresholds throughout the lactation period, respectively. Cows in which the BLV-PVL fluctuated with values above and below the cut-off threshold during lactation were excluded from the study.

3.2.4. Data collection and management

Herd-level information such as the total number of cows and animal-level information such as breed, parity, and BLV test results and Dairy Herd Improvement (DHI) data were collected by the field veterinarians in charge of the study farms via interviews with farm owners and checking farm records. Within the lactation period, event (mastitis) time was defined as the number of days from delivery until the first occurrence of mastitis. Dates of the first occurrence of mastitis were collected from DHI records of the 9 herds. Mastitis was defined as a liner score of ≥5 (283,000 somatic cell counts/ml) in the DHI tests. Cows with missing DHI records during the lactation period were excluded from the study.

Parity was categorized as 1st, 2nd and 3rd, 4th and 5th, or 6th and over. Delivery season was defined as follows: from January to March as winter, April to June as spring, July to September as summer, and October to December as fall. All data were digitized and handled using commercially available spreadsheet software (Excel 2013; Microsoft Corp., Redmond, WA, USA).

3.2.5. Survival analysis

The frailty model is a random effect survival model that allows for unobserved heterogeneity or statistical dependence between observed survival data, and random effects are treated as continuous variables that describe excess risk or frailty [87]. Frailties are useful in modeling correlations in multivariate survival and event history data, including recurrent events such as mastitis or lameness, in which an individual cow’s frailty affects the occurrence of events, and

community trials, in which different events within a community involve a common frailty (or shared frailty) shared by each individual within the community [29]. Based on my hypothesis, a two-level hierarchical causal web was constructed to illustrate the relationships between the explanatory and outcome variables (Fig. 7). Therefore, Cox regression model nested frailty was applied.

To test my hypothesis based on the data, a Cox regression model with two nested frailties (herd and cow levels) was considered. Frailty is typically defined as a clustering effect in survival analyses [15]. Two nested frailties Cox model [16, 17] can be written as follows:

$$h_{ij}\left(t|w_i w_{ij}, Z\right) = W_i W_{ij} \exp\lfloor\beta(t)Z\rfloor h_0(t) \quad \text{(Equation 7)}$$

where $h_0(t)$ represents for baseline hazard in the regression model; Z denotes the covariate vector, $\beta(t)$ represents the corresponding vector of the regression parameter, and w_i and w_{ij} represent unobserved random effects common to all observations from cow j in herd i conditional on the two nested frailties. Hazards must be positive in the Cox model, so w follows a log-normal distribution. Equation 7 can be transformed into random effects context as follows:

$$h_{ij}\left(t|u_i u_{ij}, Z\right) = \exp\left\lfloor u_i + u_{ij} + \beta(t)Z\right\rfloor h_0(t) \quad \text{(Equation 8)}$$

where $u_i = \log(w_i)$ and $u_{ij} = \log\left(w_{ij}\right)$ indicate nested random effects with zero means and variances σ_i^2 and σ_{ij}^2 for the herd and cow levels, respectively.

As shown in Figure 7, the explanatory variable of interest was BLV infection status (X). There were two potential confounders: greater parity number (C_1) can be associated with higher probabilities of BLV infection (and progression) and mastitis occurrence, whereas season of delivery (C_2) affects only mastitis occurrence.

Descriptive analyses were carried out for explanatory variables aggregated in the dataset, and

distributions and collinearities among variables were assessed. Univariable analyses of explanatory variables for the hazard of mastitis were performed using a standard Cox regression model with the Efron method for ties. The proportional hazards assumption was appraised for every predictor using Schoenfeld residuals [15].

Potentially important predictors based on the unconditional analysis results were then included in a multivariable model. Statistical associations were then inferred based on the causal web.

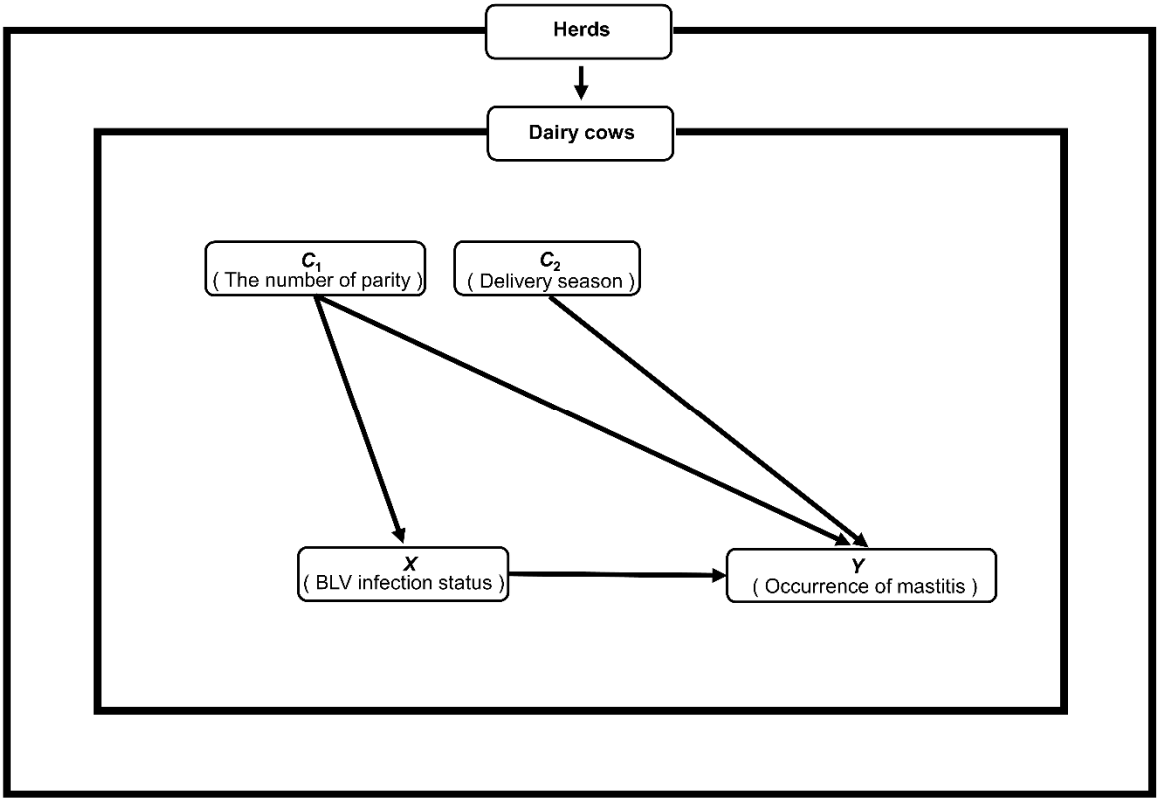

Figure 7. Theoretical causal web for the occurrence of mastitis due to BLV infection in 9 Japanese dairy herds. Two-level hierarchy affects survivorship from mastitis. The factor of interest is *X*, and the outcome is *Y*. C_1 and C_2 are potential confounders.

3.2.6. Definition of economic loss

Economic loss due to BLV infection was defined as an increase in economic loss due to increment of mastitis occurrence caused by BLV infection in a year compared to cows not infected with BLV. The definition applied to losses associated with both individual cows and Hokkaido Prefecture. Economic loss was categorized into two types:

(1) Costs of discarded milk and intra-mammary antimicrobial agents associated with treatment of BLV-induced clinical mastitis.

(2) Reduction in milk productivity due to BLV-induced sub-clinical mastitis.

In this analysis, secondary economic losses, such as increased work load, mental burden, and culling of mastitis cows, were not included.

3.2.7. Calculation procedure of economic loss

Economic loss due to BLV-associated mastitis across Hokkaido Prefecture was estimated, and these data were used to calculate the loss per individual cow for both H-PVL and non–H-PVL cows, including cows not infected with BLV. In the estimation process, Monte Carlo simulations were used to calculate some parameters, but due to the complexity of the calculation steps and lack of information regarding variations in some parameters, a point estimate approach was selected for the final estimations.

Increased economic loss due to BLV infection across Hokkaido Prefecture was estimated as follows. First, the economic loss due to clinical mastitis in H-PVL cows was estimated ($Losscmas_{HPVL}$, i). Second, the loss due to reduced milk production caused by sub-clinical mastitis in H-PVL cows was estimated ($Lossred_{HPVL}$, ii). The estimated number of H-PVL cows was used for these analyses because the hazard of L-PVL cows was not significantly different from that of non-infected cows but H-PVL cows (see Results section). Third, these two types of losses were summed ($Loss_{HPVL}$, iii = i + ii). Fourth, baseline losses due to clinical ($Base_{cmas}$, iv) and sub-clinical mastitis ($Base_{scmas}$, v), if these H-PVL cows were not infected

with BLV, were calculated (*Base*, vi = iv + v). Finally, the baseline economic loss (vi) was subtracted from the economic loss for H-PVL cows (iii) to calculate the increased economic loss in Hokkaido Prefecture due to mastitis resulting from BLV infection (*Eloss*, vii = iii - vi).

To estimate the economic loss due to clinical mastitis in H-PVL cows in Hokkaido Prefecture (i), the number of H-PVL clinical mastitis cases ($Ncmas_{HPVL}$) was first estimated by multiplying the number of clinical mastitis cases in Hokkaido Prefecture in 2017 ($Ncmas$), 264,443 [36], with the estimated proportion of H-PVL cows among clinical mastitis cases in the Prefecture ($PHPVL_{cmas}$) (Equation 9).

$$Ncmas_{HPVL} = Ncmas \times PHPVL_{cmas} \quad \text{(Equation 9)}$$

The proportion of H-PVL cows among clinical mastitis cases, $PHPVL_{cmas}$, was estimated using the proportion of H-PVL cows among sub-clinical mastitis cases ($PHPVL_{scmas}$), assuming their similarity. $PHPVL_{scmas}$ was estimated by calculating the proportion of H-PVL cows among sub-clinical mastitis cows in Hokkaido Prefecture (Equation 10).

$$PHPVL_{scmas} = Nscmas_{HPVL}/(Nscmas_{HPVL} + Nscmas_{nonHPVL}) \quad \text{(Equation 10)}$$

The number of sub-clinical mastitis cases among H-PVL cows ($Nscmas_{HPVL}$) was estimated by multiplying the number of dairy cows in Hokkaido Prefecture ($Ncows$) as of 2017 (n = 496,400) [57] with the following parameters: animal-level BLV prevalence ($PrevBLV$), reported as 11.5% in the latest study [65]; proportion of H-PVL cows among BLV-infected cows in this study ($PHPVL_{overall}$), 8.3%; and the mean probability of sub-clinical mastitis occurrence among H-PVL cows ($Pscmas_{HPVL}$) (Equation 11).

$$Nscmas_{HPVL} = Ncows \times PrevBLV \times PHPVL_{overall} \times Pscmas_{HPVL} \quad \text{(Equation 11)}$$

Similarly, the number of sub-clinical mastitis cases among non-H-PVL cows (L-PVL cows and cows not infected with BLV: $Nscmas_{nonHPVL}$) was estimated using the number of non-H-PVL cows ($Ncows_{nonHPVL}$) and the mean probability of sub-clinical mastitis occurrence among non-H-PVL cows ($Pscmas_{nonHPVL}$) (Equation 12):

$$Nscmas_{nonHPVL} = Ncows_{nonHPVL} \times Pscmas_{nonHPVL} \quad \text{(Equation 12)}$$

where $Ncows_{nonHPVL}$ represents the value resulting from subtracting the number of H-PVL cows ($Ncows \times PrevBLV \times PHPVL_{overall}$ in Equation 11) from $Ncows$.

To estimate the probabilities of sub-clinical mastitis for H-PVL ($Pscmas_{HPVL}$) and non-HPVL ($Pscmas_{nonHPVL}$) cows, respectively, the proportion of sub-clinical mastitis cows was randomly sampled based on Kaplan–Meier survival curves for sub-clinical mastitis in H-PVL and non-H-PVL cows at 310 days post calving; therefore, these proportions represent the prevalence of sub-clinical mastitis at any time point. The Monte Carlo simulations were iterated 5,000 times for both H-PVL and non-H-PVL cows. I selected 310 days post calving because in 2017, the calving interval mode for dairy cows in Hokkaido Prefecture was 358 days, and the dry period was approximately 60 days [48], resulting in 298 days in milking; considering cows for which the calving interval was not calculated due to replacement, the milking period was set longer.

To estimate the cost of clinical mastitis per cow ($Losscmas_{cow}$), it was assumed that milk was discarded (i.e., wasted) for 7 days (Day_{waste}) for a single clinical mastitis treatment. Based on an average amount of 305-day milk of 9,626 kg in Hokkaido Prefecture in November 2017 [48], the average daily milk production per head was 31.6 kg (Vol_{day}). The raw milk unit was assumed to be 100 yen per kg ($Price_{milk}$), and the cost of intra-mammary antimicrobials used to treat mastitis was 100 yen (equivalent to 0.97 US dollars based on the 2020 November 6

exchange rate of 103.5 yen, $Price_{AM}$) per day. Mastitis treatment was assumed to be continued for 3 days (Day_{treat}).

$$Losscmas_{cow} = Vol_{day} \times Day_{waste} \times Price_{milk} + Day_{treat} \times Price_{AM} \quad \text{(Equation 13)}$$

Finally, the economic loss due to clinical mastitis in H-PVL-cows in Hokkaido Prefecture ($Losscmas_{HPVL}$, i) was calculated from the product of the number of clinical mastitis cases in H-PVL cows ($Ncmas_{HPVL}$) and the unit cost ($Losscmas_{cow}$) (Equation 14).

$$Losscmas_{HPVL} = Ncmas_{HPVL} \times Losscmas_{cow} \quad \text{(Equation 14)}$$

To estimate the loss due to reduced milk production caused by sub-clinical mastitis associated with BLV infection ($Lossred_{HPVL}$, ii), the mean decline in milk production of a cow with sub-clinical mastitis was multiplied with the total number of milking days under sub-clinical mastitis conditions of affected cows in Hokkaido Prefecture (Equation 15). For this calculation, the mean number of milking days per cow in 1 year (Day_{milk}) was first estimated by randomly sampling 5,000 times under the scenario of the number of days milked by the end of the year (December 31) for a series of 365 cows with different delivery days starting January 1 and continuing through December 31, assuming that all of the cows were milked for 310 days. This process indicated that on average, a single cow is milked 178.0 days per year. Second, the number of days milked under sub-clinical mastitis conditions (Day_{scmas}) was estimated by multiplying the number of milking days per year with the proportion of days milked under sub-clinical mastitis conditions for H-PVL-cows ($Pdayscmas_{HPVL}$). $Pdayscmas_{HPVL}$ was calculated from the time since delivery at the median survival proportion for sub-clinical mastitis among H-PVL cows. Half of mastitis cases occur before 30 days, meaning that cows have mastitis or are in the recovery stages after treatment for 90.3% [(310 – 30 days)/310 days]

of the lactation period. The rate of reduction in milk production among cows with sub-clinical mastitis (Red_{scmas}) was estimated by taking the complement to 1 of the ratio of the average milk yield over 305 days for cows with a linear score of ≥5 to that of healthy cows. Red_{scmas} was 5.0%, indicating a reduction per cow per day of 1.675 kg. This calculation assumed that the reduction in milk production continues even after mastitis treatment, and this may be a strong assumption.

$$Lossred_{HPVL} = Nscmas_{HPVL} \times Vol_{day} \times Price_{milk} \times Day_{milk} \times Pdayscmas_{HPVL} \times (1 - Red_{scmas}) \quad \text{(Equation 15)}$$

The baseline loss due to clinical mastitis ($Base_{cmas}$, iv) among H-PVL cows (the loss if these cows did not exhibit H-PVL status) was estimated by applying the incidence rate of clinical mastitis in non-H-PVL-cows to the estimated H-PVL bovine population ($Ncmas_{HPVL}$).

The baseline loss due to sub-clinical mastitis ($Base_{scmas}$, v) among H-PVL cows (again, the loss if these cows did not exhibit H-PVL status) was estimated by applying the proportion of sub-clinical mastitis cows among non-H-PVL cows described above ($Pscmas_{nonHPVL}$) and the median number of days under sub-clinical mastitis conditions among non-H-PVL cows to the H-PVL bovine population ($Ncmas_{HPVL}$) (Equation 16). The number of days under sub-clinical mastitis condition among non-H-PVL cows was estimated by the same approach with the H-PVL cows. Non-H-PVL cows had mastitis or were after treatment in 64.2% of the lactation period. The number of days under sub-clinical mastitis conditions among non-H-PVL cows was estimated using the same approach with H-PVL cows. Non-H-PVL cows had mastitis or were in the post-treatment state for 64.2% of the lactation period.

$$Base_{scmas} = Ncmas_{HPVL} \times Vol_{day} \times Price_{milk} \times Day_{milk} \times Pdayscmas_{nonHPVL} \times (1 - Red_{scmas}) \quad \text{(Equation 16)}$$

The economic loss due to BLV-associated mastitis per H-PVL cow was estimated by dividing the loss in Hokkaido Prefecture ($Loss_{HPVL}$) by the estimated number of H-PVL cows (Equation 17).

$$Loss_{HPVLcow} = Loss_{HPVL}/(Ncows \times PrevBLV \times PHPVL_{overall}) \quad \text{(Equation 17)}$$

For non-H-PVL cows, first, the economic losses by clinical and sub-clinical mastitis were separately estimated for Hokkaido Prefecture, and then it was divided by the number of non-HPVL cows. The economic loss due to clinical mastitis in non-H-PVL cows in Hokkaido Prefecture was estimated by multiplying the number of clinical mastitis cases among non-H-PVL cows estimated as Equation 18 with the unit loss per cow ($Losscmas_{cow}$) estimated in Equation 13 (Equation 19).

$$Ncmas_{nonHPVL} = Ncmas - Ncmas_{HPVL} \quad \text{(Equation 18)}$$

$$Losscmas_{nonHPVL} = Ncmas_{nonHPVL} \times Losscmas_{cow} \quad \text{(Equation 19)}$$

The economic loss in non-H-PVL cows due to sub-clinical mastitis in Hokkaido Prefecture was estimated by multiplying the number of sub-clinical mastitis cases in non-PVL cows ($Nscmas_{nonHPVL}$) estimated in Equation 6 with the unit loss for non-HPVL cows (Equation 20).

$$Lossred_{nonHPVL} = Nscmas_{nonHPVL} \times Vol_{day} \times Price_{milk} \times Day_{milk} \times Pdayscmas_{nonHPVL} \times (1 - Red_{scmas}) \quad \text{(Equation 20)}$$

The baseline economic loss due to mastitis per non-H-PVL cow ($Loss_{nonHPVLcow}$) was estimated by dividing the sum of the two abovementioned losses by the number of non-H-PVL cows in Hokkaido Prefecture (Equation 21).

$$Loss_{nonHPVLcow} = (Losscmas_{nonHPVL} + Lossred_{nonHPVL})/Ncmas_{nonHPVL}$$

(Equation 21)

Using the proportion of H-PVL cows among BLV-infected cows in my dataset (i.e., 8.2%), the difference in economic loss between H-PVL and L-PVL cows was weighted, and the averaged loss per BLV-infected cow ($Loss_{BLVcow}$) was calculated. Finally, the increased cost of mastitis associated with H-PVL status at the individual cow level ($Eloss_{cow}$) was estimated using Equation 22.

$$Eloss_{cow} = Loss_{HPVLcow} - Loss_{nonHPVLcow}$$ (Equation 22)

All statistical analyses were performed using R software, version 3.6.0 and R studio, version 1.2.5042 [96, 97]. R package coxme, version 2.2-16, was used for survival analyses [98].

3.3. Results

3.3.1. Descriptive Statistics

The farms included in the study reared between 57 and 284 cows, with a mean and median of 133 and 87 cows, respectively. The proportion of cows within a herd diagnosed with mastitis ranged between 18.8% and 56.5%, with a mean and median of 41.1% and 48.2%, respectively. The total number of cows studied was 1,034, which included 868 cows not infected with BLV, 135 L-PVL cows, and 31 H-PVL cows. The overall proportion of cows diagnosed with mastitis during the study period in the 9 herds examined was 42.6% (440/1,034), and the proportions of cows diagnosed with mastitis within 50, 110, and 210 post-partum days were 15.0%, 25.0%, and 36.7%, respectively. The median day of mastitis diagnosis and censoring day were 92 and 263.5, respectively.

The descriptive statistics for cow-level predictor variables included in the analysis are shown in Table 9. Kaplan–Meier survival curves for mastitis events by BLV infection status (non-infected, L-PVL, and H-PVL) and parity (1st, 2nd and 3rd, 4th and 5th, and 6th and over) are shown in Figure 8.

In log-rank tests for multi-collinearity, the *p*-values for three predictors (BLV infection status, parity, and delivery season) were <0.2, suggesting no collinearity.

Table 9. Descriptive summary of cows and farms studied for BLV infection and mastitis between April 2015 and March 2018.

Variables	Number of cows	Proportion of cows diagnosed with mastitis (%)
BLV infection status		
Non-infected	868	40.7
Low-proviral-load	135	45.9
High-proviral-load	31	80.6
Parity		
1st	366	29.6
2nd and 3rd	431	44.8
4th and 5th	170	56.5
6th and over	67	64.2
Delivery season		
Spring	259	45.9
Summer	307	45.6
Fall	237	39.2
Winter	231	38.2
Herd		
A	23	56.5
B	99	48.5
C	186	53.8
D	127	18.9
E	83	48.2
F	69	40.6
G	72	19.7
H	304	48.7
I	71	35.2

Low-proviral-load and High-proviral-load cows were defined as those in which the proviral load was below and above the cut-off threshold through the lactation period, respectively.

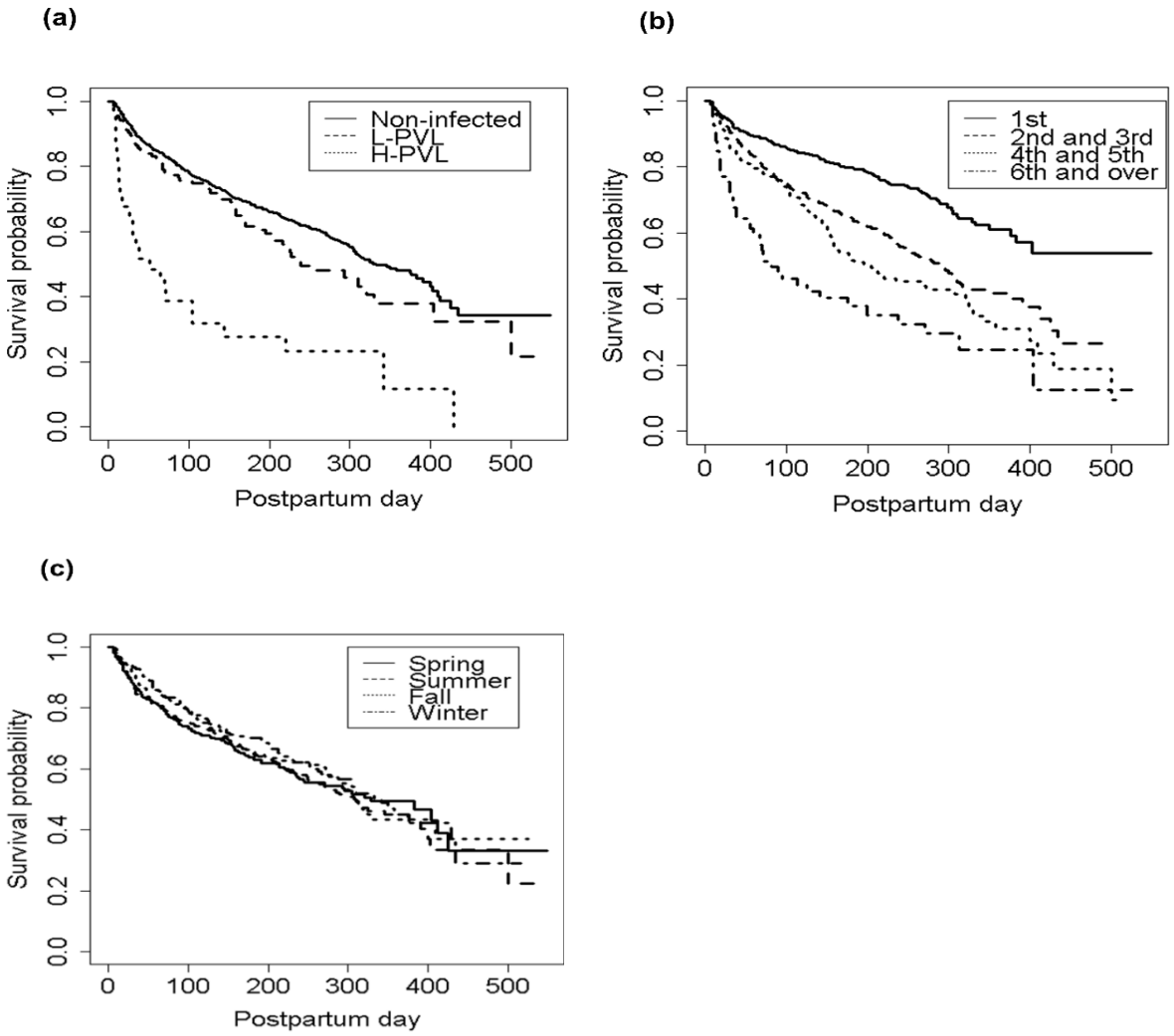

Figure 8. Kaplan–Meier survivor curves for mastitis occurred between April 2015 and March 2018. The panels (a), (b), and (c) show the comparisons between BLV infection status, the numbers of parity, and seasons, respectively.

3.3.2. Multivariable analysis

A multivariable analysis was performed based on BLV infection status (non-infected, L-PVL, and H-PVL), parity (1st, 2nd and 3rd, 4th and 5th, and 6th and over), and delivery season (spring, summer, fall, or winter). Figure 7 suggests the possibility of an interaction between X and C_1. However, Kaplan–Meier plots did not suggest an existing interaction, and the multivariable model did not include the interaction term. Results of the multivariable model is tabulated in Table 10. The HR for mastitis for H-PVL cows was 2.6 times higher than that for cows not infected with BLV. The HR of mastitis increased with parity number. Delivery season was not associated with the HR of mastitis.

Table 10. Results of multivariable survival analysis for mastitis among 9 herds between April 2015 and March 2018.

Predictor	Hazard ratio	Standard error	*p*-value
Fixed effect			
BLV infection status			
Non-infected	Reference		
Low-proviral-load	0.79	0.18	0.18
High-proviral-load	2.61	0.28	<**0.01**
Parity			
1st	Reference		
2nd and 3rd	2.10	0.13	<**0.01**
4th and 5th	2.81	0.17	<**0.01**
6th and over	5.45	0.22	<**0.01**
Delivery season			
Spring	Reference		
Summer	1.05	0.14	0.72
Fall	0.90	0.16	0.50
Winter	0.86	0.16	0.34
Random effect	Variance	Standard deviation	
Between-cows variance	0.37	0.61	
Between-herd variance	0.26	0.51	

Low-proviral-load and high-proviral-load cows were defined as those in which the proviral load was below and above the cut-off threshold throughout the lactation period, respectively.

3.3.3. Estimation for annual economic losses

The estimated numbers of H-PVL and non-H-PVL cows in Hokkaido Prefecture were 4,721 and 4,901,679, respectively. The estimated prevalences of sub-clinical mastitis in H-PVL and non-H-PVL cows were 65.8% [95% credible interval (CI): 62.3-68.9%] and 29.7% (95% CI: 27.0-32.3%), respectively (Fig. 9). Based on the prevalence data, the estimated numbers of sub-clinical mastitis H-PVL and non-H-PVL cows at any given time were 3,107 and 145,957, respectively. Based on these data, the estimated proportion of H-PVL cows among sub-clinical mastitis cases was 2.1%. Finally, the estimated annual incidences of clinical mastitis in H-PVL and non-H-PVL cows in Hokkaido Prefecture were 5,553 and 258,890 cases, respectively.

Table 11 summarizes the economic loss due to mastitis associated with BLV infection. The annual economic loss due to mastitis in H-PVL cows in Hokkaido Prefecture (iii in Table 11) was almost 2 million US dollars. By subtracting the baseline loss—the loss which would have occurred even in the absence of BLV infection—in these cows (vi), the estimated increased loss due to BLV-associated mastitis in Hokkaido Prefecture (vii) was 1.2 million US dollars.

At the individual-cow level, the annual loss due to mastitis per H-PVL cow (416 US dollars) was 2.5 times greater than that per non-H-PVL cow (166 US dollars). The increased cost of BLV-associated mastitis per cow (250 US dollars) demonstrates the magnitude of economic impact; it was even greater than the baseline loss due to mastitis per non-H-PVL cow (166 US dollars).

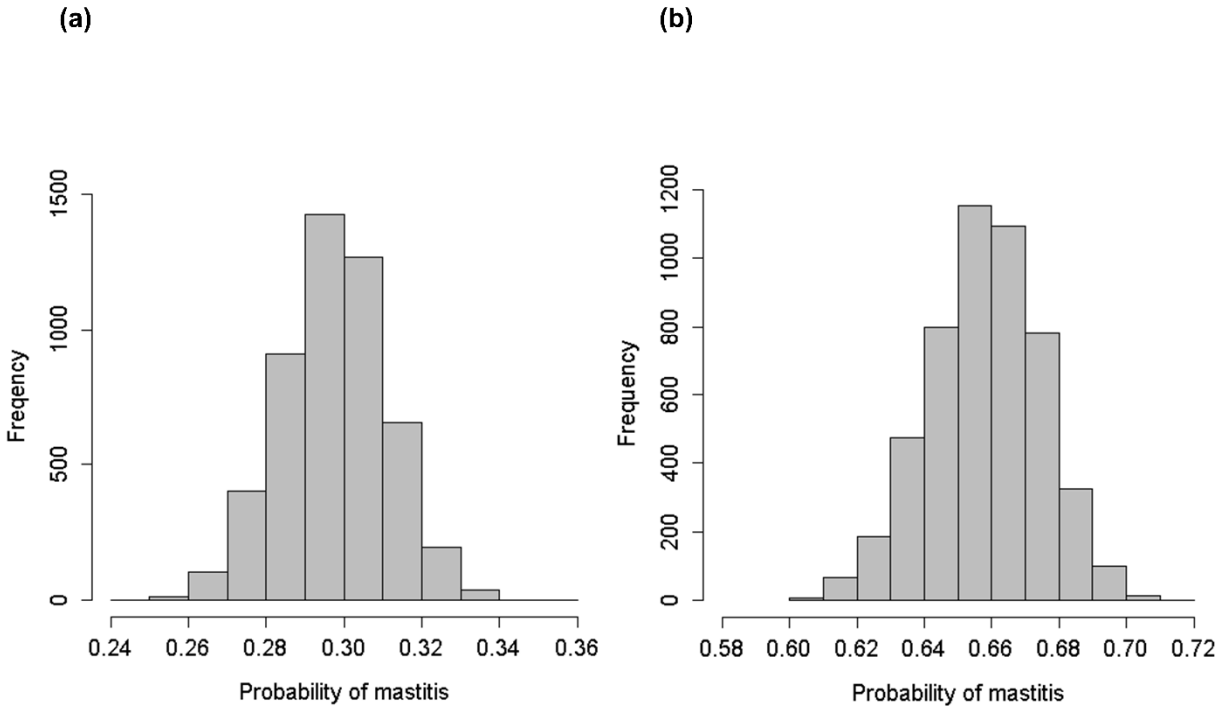

Figure 9. Results of bootstrap analysis of mastitis prevalence. Panel (a) shows the histogram among BLV non-infected cows, while panel (b), that of H-PVL cows.

Table 11. Estimations of economic losses due to mastitis associated with BLV infection in Hokkaido Prefecture

Notation	Description	Value (USD)	Value (JPY)
$Losscmas_{HPVL}$ (i)	Loss due to clinical mastitis in H-PVL cows in Hokkaido Prefecture	1,202,947	124,505,053
$Lossred_{HPVL}$ (ii)	Loss due to reduced milk production caused by sub-clinical mastitis in H-PVL cows in Hokkaido Prefecture	762,298	78,897,844
$Loss_{HPVL}$ (iii): (i) + (ii)	Loss due to mastitis in H-PVL cows in Hokkaido Prefecture	1,965,245	203,402,897
$Base_{cmas}$ (iv)	Baseline loss due to clinical mastitis in H-PVL cows in Hokkaido Prefecture	538,473	55,731,905
$Base_{scmas}$ (v)	Baseline loss due to sub-clinical mastitis in H-PVL cows in Hokkaido Prefecture	244,631	25,319,271
$Base$ (vi): (v) + (vi)	Baseline loss due to mastitis in H-PVL cows in Hokkaido Prefecture	783,103	81,051,176
Eloss (vii): (iii) – (vi)	Elevated loss in mastitis due to BLV infection in Hokkaido Prefecture	1,182,142	122,351,721

Table 11. (continued)

Notation	Description	Value (USD)	Value (JPY)
$Loss_{HPVLcow}$	Loss due to mastitis per H-PVL cow	416	43,085
$Loss_{BLVcow}$	Loss due to mastitis per BLV-infected cow	192	19,841
$Loss_{nonHPVLcow}$	Baseline loss due to mastitis per non-H-PVL cow	166	17,172
$Eloss_{cow}$	Elevated cost of mastitis associated with BLV infection per cow	250	25,912

USD, US dollars; JPY, Japanese yen.

3.4. Discussion

Mastitis is an inflammation of the mammary gland caused primarily by bacterial infection. Typically, 72.8% of cows with mastitis recover and remain in the herd, whereas 24% are removed or sold [66]. Risk factors for mastitis have been widely investigated [24, 46]. However, the relationship between BLV infection and the occurrence of mastitis remains unclear. To our knowledge, this study is the first to report that BLV infection in H-PVL cows is associated with the occurrence of mastitis.

I hypothesized that BLV suppresses immune function, leading to an increased incidence of mastitis. Numerous researchers have reported that BLV infection reduces milk productivity [6, 18, 68, 72, 79], but the mechanism has not been elucidated. The Kaplan–Meier survivor curves constructed in the present study demonstrated that a half of H-PVL cows suffered from mastitis within 52 days after calving, and among the mastitis cases observed within 310 days after delivery, one-half occurred within 30 days. Milk fever and ketosis, which frequently occur during the post-partum period until peak lactation, are associated with nutritional management [23]. Negative energy balance from the post-partum period to peak lactation inhibits immune function [93]. In addition to these known factors, my survival analysis results identified H-PVL status as a significant risk factor for the mastitis, which reduces milk productivity. Several previous studies reported the mechanism of immunosuppression in BLV infection. BLV is harbored in the mammary glands of BLV-infected cows with sub-clinical mastitis [111], where it can cause immunosuppression. A higher percentage of $CD5^{-}/CD11b^{-}$ B cells in the milk of BLV-infected cows with persistent lymphocytosis compared with non-infected or BLV-infected aleukemic cows has been reported, indicating dysfunction of milk neutrophils [14]. Moreover, the concentration in milk of lingual antimicrobial peptide, a natural immunity factor that is indicative of immune function in the mammary gland, is lower in H-PVL cows than L-PVL cows [107].

The survival analysis of the present study identified another significant factor associated with

mastitis: parity number. The incidence of mastitis reportedly increases with increase in parity number [32, 112]. In contrast, unlike previous studies [44, 77], delivery season was not associated with the occurrence of mastitis in my study. The study area was located near 43° north latitude, which is characterized by a cool climate. The cows on the farms studied are therefore less likely to undergo heat stress during the summer, thus reducing the likelihood of seasonal mastitis.

Several farm-level factors, such as farm dairy hygiene [22, 62], and individual animal-level factors, such as sensitivity of mastitis [52] are known to affect the occurrence of mastitis. However, in my study employed a frailty model to adequately control for such clustering effects [86].

This was the first economic study of BLV-associated mastitis in Japan. The annual economic loss in H-PVL cows was 2.51 times higher than that in non-H-PVL cows, suggesting there is economic merit in removing H-PVL cows from the herd. The estimated annual economic loss in BLV-infected cows was 191.7 US dollars. According to a study in the USA, BLV-seropositive cows produced 59 US dollars less annually than non-infected cows [79]. The method for calculating economic losses was different in these studies, but considering the difference in raw milk unit prices between the USA (raw milk unit price per kg in the USA was 0.39 US dollars as of 2020 September 30) and Japan (0.97 US dollars per kg), my estimate of the annual economic loss in BLV-infected cows appears reasonable. The estimated loss due to early lactation clinical mastitis in a previous study was 444 US dollars [85], approximately the same as the loss in H-PVL cows in the present study. This was consistent with survival curve data indicating that many H-PVL cows develop mastitis during early lactation (Fig. 8 a). Isolation and culling of H-PVL cows are effective methods for decreasing BLV infection because they reduce the likelihood of new BLV infection [88]. However, in the absence of a national eradication scheme with compensation programs, motivation for BLV control is generally low among Japanese dairy farmers. Mastitis is a common disease in the dairy industry

and the most common reason for culling [67] ; the fact that half of H-PVL cows have mastitis from the post-partum period until peak lactation suggests that economic losses are enormous. This message should provide clear incentive for owners to cull H-PVL cows earlier and thus prevent the spread of BLV infection on their dairy farms.

This research has 3 limitations due to the availability of data. First, I only assumed that clinical mastitis was treated using intra-mammary antimicrobial agents; however, treatment of clinical mastitis included veterinary care. The cost of veterinary services was not calculated because the system in Japan involves publicly funded livestock insurance. Therefore, the economic losses estimated in this study may be underestimated. Mastitis was the second most-common reason for dairy cow removal in Hokkaido Prefecture in 2017, as approximately 10% of mastitis-affected cows were culled [48]. However, this economic study did not assume the cost of replacing culled cows, which could have led to further underestimation of economic losses. Third, the reduction in milk productivity was modeled to begin at the occurrence of sub-clinical mastitis and continue throughout the lactation period; thus, these losses could be slightly overestimated.

In conclusion, this study revealed that BLV-infected H-PVL cows exhibit a higher HR for mastitis after adjustment for parity number, delivery season, and clustering effects at the farm and individual-cow levels. BLV-infected H-PVL cows are associated with significant economic losses in Hokkaido Prefecture. Priority removal of BLV-infected HPVL cows is recommended in terms of both BLV infection control and economics.

3.5. Summary

Bovine leukemia virus (BLV), which causes enzootic bovine leucosis and immunosuppression, is widely prevalent on Japanese dairy farms. However, in the absence of a national eradication scheme with compensation programs, it is important to estimate BLV-associated economic losses to raise farmers' awareness. Mastitis is a common disease in the dairy industry and the most common reason for culling. I hypothesized that immunosuppression due to BLV causes mastitis. A retrospective cohort study was conducted to trace Holstein cows at 9 commercial dairy farms in the Nemuro and Kushiro regions of Hokkaido Prefecture, Japan, where monitoring of BLV proviral load (PVL) is routine. Information regarding Dairy Herd Improvement data, parity number, and delivery day were collected at each farm. Cows with no confirmed infection during lactation were defined as non-infected. Low-proviral-load (L-PVL) and high-proviral-load (H-PVL) cows were defined as those in which PVL was below and over 2,465 copies/50 ng DNA, respectively, or 56,765 copies/10^5 cells, respectively, throughout the lactation period. Survival analysis was performed using the frailty model to estimate the hazard ratio (HR) of mastitis for BLV infection status using data from 1,034 dairy cows after adjusting for known confounding factors. Using field and published data, annual economic losses were estimated using Monte Carlo simulation. Kaplan–Meier survivor curves demonstrated that half of the H-PVL cows developed mastitis within 52 days after calving. The HR of mastitis for H-PVL cows was 2.61 times higher than that of non-infected cows. In 2017, there were 264,443 clinical mastitis cases in Hokkaido. The economic loss due to mastitis associated with BLV infection per H-PVL cow was 43,085 yen and 416 US dollars, with the annual economic loss in Hokkaido Prefecture due to mastitis caused by BLV infection estimated at 122,351,721 yen and 1,182,142 US dollars. In summary, H-PVL cows are more susceptible to mastitis than non-infected and L-PVL cows. Mastitis due to BLV infection is thus associated with significant

economic losses.

General discussion

Highlights of the thesis

In this thesis, a neglected infectious disease of dairy cows: bovine leukemia was investigated using epidemiology and animal health economics. In Japan, BLV infection continues to spread [64, 65], and the number of EBL notifications became the second-largest after Johne's disease [55]. Therefore, in 2015, the Ministry of Agriculture, Forestry, and Fisheries (MAFF) of Japan published ‘Guidelines for Biosecurity Measures of Enzootic Bovine Leukosis’ [53]. However, due to the lack of compensation for culling of BLV infected cows, efforts to the disease control is limited.

In Chapter 1, establishment of a quantitative procedure for estimating blood PVL/μl using a statistical model was attempted. In the MAFF’s guideline for BLV control, the “test and segregate” or “test and manage” strategy is recommended, and diagnostic methodologies such as ELISA, qualitative PCR, and quantitative real-time PCR were introduced. However, the methodologies to follow were not strictly specified, and there is still a room to discuss cost effectiveness in choosing the method. Real-time PCR and ELISA are widely used in Japan to diagnose with BLV infected dairy cows [72], but they can be costly in testing large number of cows My prediction method offers a cost-effective alternative to the conventional methods. In fact in Europe, the EC ‘s key was used as an indicator to cull potentially BLV-infected cows and eradication of BLV has been achieved. The EC ‘s key uses the age and lymphocyte count of cows. My model further improved the EC’ s key because it estimates BLV PVL using these information. Priority culling of H-PVL cows has been reported to efficiently reduce BLV transmission as well as prevalence [88]. My model does not require expensive measurement equipment and allow identifying potential H-PVL cows for priority culling.

In Chapter 2, the economic loss due to BLV-associated carcass weight reduction of dairy cows was estimated. Dairy cows are an important source of beef [56], and quantification of such economic loss is important in understanding the impact of BLV. The number of EBL cattle condemned at meat inspection in slaughterhouses has been increasing in Japan due to the

increase in BLV prevalence [92], hence the economic importance is increasing. A hypothesis that decreased immune function due to BLV infection would increase visceral abnormalities, in turn reducing carcass weight was formulated, and was tested using mediation analysis. As a result, this study revealed that a mean carcass weight for H-PVL culled cows was significantly lighter, by 30.2 kg than that of non-infected cows. When AFPE is increased by 1, carcass weight significantly decreased by 8.55 kg. The mediation analysis indicated that the indirect effect was significant for H-PVL culled cows, whereas not significant for L-PVL. The proportion of the effect mediated by AFPE was 21.6% for H-PVL culled cows, a value that was much larger than the 8.7% obtained for L-PVL culled cows. The median annual direct loss of carcass weight due to BLV infection in Hokkaido Prefecture in 2017 was estimated to be 118,166 kg, and the annual economic loss was 66,172,680 yen, which was 626,277 US dollars.

In this study, variables and analysis methods were determined based on the hypothetical causal web. This suggested the importance of drawing a causal web in epidemiology and animal health economics as well.

In Chapter 3, the economic loss due to BLV- associated mastitis in Hokkaido Prefecture was estimated. Mastitis is a major economic constraint in dairy industry, and 72.8% of cows with mastitis recover and remain in the herd, while 24% are removed or sold [67]. In 2017, there were about 265,000 cases of clinical mastitis in Hokkaido [36]. The hypothesis in this study was that suppressed immune function due to BLV infection would increase the incidence of mastitis. As far as I know, no study was conducted to infer the association between BLV infection status and occurrence of mastitis, and to estimate the annual economic loss of BLV-associated mastitis. The hazard ratio for mastitis for H-PVL cows was 2.6 times higher than that of BLV-non-infected cows. The Kaplan–Meier survivor curves proved that a half of H-PVL cows suffered from mastitis within 52 days after calving, and among the mastitis observed within 310 days since delivery, a half occur within 30 days. The economic loss due to mastitis associated with BLV infection per H-PVL cow was 43,0845 yen and 416 US dollars, and the

annual economic loss due to mastitis caused by BLV infection in Hokkaido Prefecture was estimated to be 122,351,721 yen, which is 1,182,142 US dollars. Overall, my study revealed the high economic loss due to BLV infection on the dairy production in Hokkaido Prefecture.

Integration of the studies

The three components of this thesis are strongly related, in delivering a key message of the importance of identifying and culling H-PVL cows. The first study established a cost-effective alternative to costly conventional methods, and this simple method can be powerful in rapidly and cheaply identifying H-PVL cows in a herd. The second and third chapter showed how damages in immune systems due to BLV affect the productivity, and how much money the dairy industry are losing by simply allowing H-PVL cows to stay and spread BLV in dairy herds in Japan. This thesis provided enough information to convince the dairy industry and the government to accelerate the policy development and voluntary efforts in BLV control.

Further perspectives of the economic loss associated with bovine leukemia virus infection

This thesis attempted to quantify the economic loss due to BLV in beef production using culled dairy cows and mastitis. There are other components in completing the BLV-associated economic loss in dairy industry in Hokkaido Prefecture, namely, whole condemnation of carcasses due to malignant lymphoma, and reproduction failure. Together with these losses, future study should quantify total economic loss incurred due to BLV infection in dairy cows in Hokkaido Prefecture. Such complete economic evaluation will lead to the cost-effectiveness discussions of BLV control strategies. At this stage, economic evaluation in disease control should require policy design discussions including the cost of equipment and materials needed in the control and financial aid planning for dairy farmers.

As to more on epidemiological side, an association between the occurrence of mastitis and

fertility has been noted during clinical services, and mediation analysis may infer the causality between infection with BLV, occurrence of mastitis, and the fertility. The approaches described in this thesis should be well applicable to solve problems of the dairy industry in Hokkaido, Japan, including other important diseases such as Johne’s disease. The efforts of applying epidemiology and animal health economics will be surely continued to support the industry.

Acknowledgements

Firstly, I would like to express my appreciation to my supervisor, Professor Kohei Makita, Head of Veterinary Epidemiology Unit, Graduate School of Veterinary Medicine, Rakuno Gakuen University Graduate School for his continuous support and guidance throughout my PhD study. His motivation as a veterinary epidemiologist was a large factor that I decided to study veterinary epidemiology.

I would also like to gratefully thank Dr. Junko Kohara, Dr. Takeshi Miyama and Dr. Yuri Fujimoto for their support, advices, comments, encouragement, and friendship.

I would like to thank Dr. Yasumoto Adachi for their insightful advices for the study about Chap.2. I thank the Hokkaido Government for providing the postmortem examination records. I much appreciate the participating farmers for their contributions. I thank the BLV control committee of the Hokkaido Higashi Agriculture Mutual Aid Association.

I would like to thank Hokkaido Research Organization for funding the study about BLV. I would like to thank the farms provided the data for their contributions.

I would like to thank my fellows and laboratory mates in Veterinary Epidemiology Unit for insightful discussions and their friendship. My life during my PhD course with them was a cheerful one.

Finally, I would like to thank my wife and my daughters for their deep supports and encouragements during my PhD course.

References

1. Acaite, J., Tamosiunas, V., Lukauskas, K., Milius, J. and Pieskus, J. 2007. The eradication experience of enzootic bovine leukosis from Lithuania. *Prev. Vet. Med.* **82**: 83-89.
2. Adachi, Y. and Makita, K. 2015. Real time detection of farm-level swine mycobacteriosis outbreak using time series modeling of the number of condemned intestines in abattoirs. J Vet Med Sci 77: 1129-1136.
3. Adachi, Y. and Makita, K. 2017. Time series analysis based on two-part models for excessive zero count data to detect farm-level outbreaks of swine echinococcosis during meat inspections. Prev Vet Med 148: 49-57.
4. Alvarez, I., Gutierrez, G., Gammella, M., Martinez, C., Politzki, R., Gonzalez, C., Caviglia, L., Carignano, H., Fondevila, N., Poli, M. and Trono, K. 2013. Evaluation of total white blood cell count as a marker for proviral load of bovine leukemia virus in dairy cattle from herds with a high seroprevalence of antibodies against bovine leukemia virus. *Am J Vet Res* **74**: 744-749.
5. Amirpour Haredasht, S., Vidal, G., Edmondson, A., Moore, D., Silva-Del-Rio, N. and Martinez-Lopez, B. 2018. Characterization of the Temporal Trends in the Rate of Cattle Carcass Condemnations in the US and Dynamic Modeling of the Condemnation Reasons in California With a Seasonal Component. *Frontiers in veterinary science* **5**: 87.
6. Bartlett, P. C., Norby, B., Byrem, T. M., Parmelee, A., Ledergerber, J. T. and Erskine, R. J. 2013. Bovine leukemia virus and cow longevity in Michigan dairy herds. *Journal of dairy science* **96**: 1591-1597.

7. Bartlett, P. C., Sordillo, L. M., Byrem, T. M., Norby, B., Grooms, D. L., Swenson, C. L., Zalucha, J. and Erskine, R. J. 2014. Options for the control of bovine leukemia virus in dairy cattle. *Journal of the American Veterinary Medical Association* **244**: 914-922.

8. Bates, D., Mächler, M., Bolker, B. and Walker, S. 2014. Fitting linear mixed-effects models using lme4. *arXiv preprint arXiv:1406.5823*.

9. Bürkner, P.-C. 2017. Advanced Bayesian multilevel modeling with the R package brms. *arXiv preprint arXiv:1705.11123*.

10. Carson, D. A., Barkema, H. W., Naushad, S. and De Buck, J. 2017. Bacteriocins of Non-aureus Staphylococci Isolated from Bovine Milk. *Appl Environ Microbiol* **83**: e01015-01017.

11. Crawley, M. J. 2013a. Generalized linear models. Pages 557–578 in The R Book. 2nd ed. John Wiley & Sons, Ltd., West Sussex, UK.

12. Crawley, M. J. 2013b. Count data. Pages 579–598 in The R Book. 2nd ed. John Wiley & Sons, Ltd., West Sussex, UK.

13. Chi, J., Weersink, A., VanLeeuwen, J. A. and Keefe, G. P. 2002. The Economics of Controlling Infectious Diseases on Dairy Farms. *Canadian Journal of Agricultural Economics/Revue canadienne d'agroeconomie* **50**: 237-256.

14. Della Libera, A. M. M. P., de Souza, F. N., Batista, C. F., Santos, B. P., de Azevedo, L. F. F., Sanchez, E. M. R., Diniz, S. A., Silva, M. X., Haddad, J. P. and Blagitz, M. G. 2015. Effects of bovine leukemia virus infection on milk neutrophil function and the milk lymphocyte profile. *Vet Res* **46**: 2-2.

15. Dohoo, I., Martin, W. and Stryhn, H. 2009. Modelling survival data. Pages 467–528 in Veterinary Epidemiologic Research, 2nd ed. VER, Inc., Prince Edward Island, Canada.

16. Elghafghuf, A., Stryhn, H. and Waldner, C. 2014. A cross-classified and multiple membership Cox model applied to calf mortality data. *Prev Vet Med* **115**: 29-38.

17. Elghafghuf, A., Dufour, S., Reyher, K., Dohoo, I. and Stryhn, H. 2014. Survival analysis of clinical mastitis data using a nested frailty Cox model fit as a mixed-effects Poisson model. *Prev Vet Med* **117**: 456-468.

18. Erskine, R. J., Bartlett, P. C., Byrem, T. M., Render, C. L., Febvay, C. and Houseman, J. T. 2012. Herd-level determinants of bovine leukaemia virus prevalence in dairy farms. *J. Dairy Res.* **79**: 445-450.

19. Fox, J. and Monette, G. 1992. Generalized Collinearity Diagnostics. *Journal of the American Statistical Association* **87**: 178-183.

20. Frie, M. C. and Coussens, P. M. 2015. Bovine leukemia virus: a major silent threat to proper immune responses in cattle. *Vet Immunol Immunopathol* **163**: 103-114

21. Frie, M. C., Sporer, K. R. B., Benitez, O. J., Wallace, J. C., Droscha, C. J., Bartlett, P. C. and Coussens, P. M. 2017. Dairy Cows Naturally Infected with Bovine Leukemia Virus Exhibit Abnormal B- and T-Cell Phenotypes after Primary and Secondary Exposures to Keyhole Limpet Hemocyanin. *Frontiers in veterinary science* **4**.

22. Fujimoto, Y., Ito, H., Higuchi, H., Ohno, H. and Makita, K. 2020. A case-control study of herd- and cow-level risk factors associated with an outbreak of Mycoplasma mastitis in Nemuro, Japan. *Preventive Veterinary Medicine* **177**: 104946.

23. Goff, J. 2006. Major advances in our understanding of nutritional influences on bovine health. *Journal of dairy science* **89**: 1292-1301.

24. Green, M. J., Bradley, A. J., Medley, G. F. and Browne, W. J. 2007. Cow, Farm, and Management Factors During the Dry Period that Determine the Rate of Clinical Mastitis After Calving. *Journal of dairy science* **90**: 3764-3776.

25. Guo, J., Lee, D., Sakrejda, K., Gabry, J., Goodrich, B., De Guzman, J., Niebler, E., Heller, T. and Fletcher, J. 2016. rstan: R Interface to Stan. R 534: 0-3.

26. Gutiérrez, G., Alvarez, I., Politzki, R., Lomónaco, M., Dus Santos, M. J., Rondelli, F., Fondevila, N. and Trono, K. 2011. Natural progression of bovine leukemia virus infection in Argentinean dairy cattle. *Vet Microbiol* **151**.

27. Gutierrez, G., Carignano, H., Alvarez, I., Martinez, C., Porta, N., Politzki, R., Gammella, M., Lomonaco, M., Fondevila, N., Poli, M. and Trono, K. 2012. Bovine leukemia virus p24 antibodies reflect blood proviral load. *BMC veterinary research* **8**: 187.

28. Gutiérrez, G., Alvarez, I., Merlini, R., Rondelli, F. and Trono, K. 2014. Dynamics of perinatal bovine leukemia virus infection. *BMC veterinary research* **10**: 82.

29. Hanagal, D. 2006. Modeling Survival Data using Frailty Models. Pages 73–84 in The Frailty Concept, 2nd ed. Springer, Inc., 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore.

30. Häsler, B., Howe, K. S., Presi, P. and Stärk, K. D. C. 2012. An economic model to evaluate the mitigation programme for bovine viral diarrhoea in Switzerland. *Preventive Veterinary Medicine* **106**: 162-173.

31. Health, E. P. o. A. and Welfare. 2015. Enzootic bovine leukosis. *EFSA Journal* **13**: 4188.

32. Hiitiö, H., Vakkamäki, J., Simojoki, H., Autio, T., Junnila, J., Pelkonen, S. and Pyörälä, S. 2017. Prevalence of subclinical mastitis in Finnish dairy cows: changes during recent decades and impact of cow and herd factors. *Acta Veterinaria Scandinavica* **59**: 22.

33. Hokkaido Government. 2014.Hokkaido data book 2011. http://www.pref.hokkaido.lg.jp/ss/tkk/databook/0301.htm (Accessed Oct 25, 2020) (in Japanese)

34. Hokkaido Government.2020. Statistics Hokkaido agriculture and rural community 2019. http://www.pref.hokkaido.lg.jp/ns/nsi/seisakug/doukou/R1doukoutoukeihyou.pdf (Accessed Oct 25, 2020) (in Japanese)

35. Hokkaido Government Nemuro Subprefectural Bureau. 2017. Annual Statistics of Agriculture. (Accessed Sep. 5, 2017) (in Japanese)

36. HOKKAIDO-NOSAI. 2017. Records of Agricultural Insurance in 2017. Accessed Jun. 30, 2020. https://www.hknosai.or.jp/cgi-bin/index.pl?page=files&view_file=3045_11 (Accessed Oct 25, 2020) (in Japanese) http://www.nemuro.pref.hokkaido.lg.jp/ss/num/nemuronougyou.htm.

37. Japan Dairy Council. 2012. National Basic Survey of Dairy Farmers. 2012. https://www.dairy.co.jp/dairydata/kulbvq0000006r3l-att/kulbvq0000006rq5.pdf (Accessed June 27, 2019) (in Japanese)

38. Jaworski, J. P., Pluta, A., Rola-Łuszczak, M., McGowan, S. L., Finnegan, C., Heenemann, K., Carignano, H. A., Alvarez, I., Murakami, K., Willems, L., Vahlenkamp, T. W., Trono, K. G., Choudhury, B. and Kuźmak, J. 2018. Interlaboratory Comparison of Six Real-Time PCR Assays for Detection of Bovine Leukemia Virus Proviral DNA. *Journal of Clinical Microbiology* **56**: e00304-00318.

39. Jimba, M., Takeshima, S. N., Matoba, K., Endoh, D. and Aida, Y. 2010. BLV-CoCoMo-qPCR: Quantitation of bovine leukemia virus proviral load using the CoCoMo algorithm. *Retrovirology* **7**: 91.

40. Jimba, M., Takeshima, S. N., Murakami, H., Kohara, J., Kobayashi, N., Matsuhashi, T., Ohmori, T., Nunoya, T. and Aida, Y. 2012. *BLV*-CoCoMo-qPCR: a useful tool for evaluating bovine leukemia virus infection status. *BMC veterinary research* **8**: 167.

41. Juliarena, M. A., Gutiérrez, S. E. and Ceriani, C. 2007. Determination of proviral load in bovine leukemia virus-infected cattle with and without lymphocytosis. Am J Vet Res 68.

42. Kabeya, H., Ohashi, K. and Onuma, M. 2001. Host immune responses in the course of bovine leukemia virus infection. *J Vet Med Sci* **63**: 703-708.

43. Kale, M., Bulut, O., Yapkic, O., Gulay, M. S., Pehlivanoglu, F., Ata, A. and Yavru, S. 2007. Effects of subclinical bovine leukemia virus infection on some production parameters in a dairy farm in southern Turkey. *Journal of the South African Veterinary Association* **78**: 130-132.

44. Kurjogi, M. M. and Kaliwal, B. B. 2014. Epidemiology of Bovine Mastitis in Cows of Dharwad District. *International Scholarly Research Notices* **2014**: 968076.

45. Kvapilík, J., Hanuš, O., Bartoň, L., Klimešová, M. V. and Roubal, P. 2015. Mastitis of dairy cows and financial losses: An economic meta-analysis and model calculation **21**: 1092-1105.

46. Lam, T. J. G. M., van den Borne, B. H. P., Jansen, J., Huijps, K., van Veersen, J. C. L., van Schaik, G. and Hogeveen, H. 2013. Improving bovine udder health: A national mastitis control program in the Netherlands. *Journal of dairy science* **96**: 1301-1311.

47. Livestock Improvement Association of Japan. 2016. Dairy Herd Test. http://liaj.lin.gr.jp/japanese/newmilk/17/H28matome.pdf (Accessed June 1, 2020) (in Japanese)

48. Livestock Improvement Association of Japan. 2017. Dairy Herd Test Summary. http://liaj.lin.gr.jp/japanese/newmilk/18/H29matome.pdf (Accessed June 1, 2020) (in Japanese)

49. Ludecke, D. 2020. sjstats: statistical functions for regression models (version 0.17. 9).

50. Makita, K., Tobinaga, T., Kadowaki, H. and Yamamoto, H. 2013. The Effects of the Types of Post Milking Teat Disinfectants on the Occurrence and Cost of Mastitis Caused by Staphylococcus aureus among Dairy Herds in Okhotsk, Japan. *Journal of Veterinary Epidemiology* **172**: 125-131.

51. Mammerickx, M., Portetelle, D., de Clercq, K. and Burny, A. 1987. Experimental transmission of enzootic bovine leukosis to cattle, sheep and goats: infectious doses of blood and incubation period of the disease. *Leuk Res* **11**.

52. Martin, P., Barkema, H. W., Brito, L. F., Narayana, S. G. and Miglior, F. 2018. Symposium review: Novel strategies to genetically improve mastitis resistance in dairy cattle. *Journal of dairy science* **101**: 2724-2736.

53. Mekata, H., Yamamoto, M., Kirino, Y., Sekiguchi, S., Konnai, S., Horii, Y. and Norimine, J. 2018. New hematological key for bovine leukemia virus-infected Japanese Black cattle. *The Journal of veterinary medical science* **80**: 316-319.

54. Ministry of Agriculture, Forestry and Fisheries. 2015. Guidelines for biosecurity measures of enzootic bovine leukosis. https://www.maff.go.jp/j/syouan/douei/pdf/ebl_guide.pdf

55. Ministry of Agriculture, Forestry and Fisheries. 2016. Annual Statistics of Notifiable Animal Infectious Diseases (1937–2015).http://www.maff.go.jp/j/syouan/douei/kansi_densen/pdf/h27_todokede_ruinen.pdf. Accessed Sep. 5, 2017. (in Japanese)

56. Ministry of Agriculture, Forestry and Fisheries. 2018. Annual Statistics of Livestock Logistics. 2018 https://www.e-stat.go.jp/stat-search/files?page=1&layout=datalist&toukei=00500227&tstat=000001044816&cycle=7&year=20170&month=0&tclass1=000001044818&tclass2=000001117355 (Accessed Oct 25, 2020) (in Japanese)

57. Ministry of Agriculture, Forestry and Fisheries. 2018. Statistical Survey on Livestock. https://www.e-stat.go.jp/dbview?sid=0003218060 (Accessed Oct 25, 2020) (in Japanese)

58. Ministry of Agriculture, Forestry and Fisheries.. 2018b. The Survey on Livestock Products Marketing. https://www.e-stat.go.jp/stat-search/files?page=1&layout=datalist&toukei=00500227&tstat=000001044816&cycle=7&year=20170&month=0&tclass1=000001044818&tclass2=000001117355

59. Ministry of Agriculture, Forestry and Fisheries. 2020. Act on Domestic Animal Infectious Diseases Control 2020. https://elaws.e-gov.go.jp/search/elawsSearch/elaws_search/lsg0500/detail?lawId=326AC1000000166_20200701_502AC0000000016&openerCode=1 (Accessed Oct 25, 2020) (in Japanese)

60. Ministry of Agriculture, Forestry and Fisheries. 2020. Standards of Rearing Hygiene Management 2020. https://www.maff.go.jp/j/syouan/douei/katiku_yobo/k_shiyou/attach/pdf/index-91.pdf (Accessed Oct 25, 2020) (in Japanese)

61. Ministry of Health, labor and Welfare. 2017. Meat inspection data. 2017. https://www.e-stat.go.jp/stat-search/files?page=1&layout=datalist&toukei=00450192&tstat=000001024512&cycle=8&tclass1=000001119394&second2=1 (Accessed Oct 25, 2020) (in Japanese)

62. Miyama, T., Joseph, B., Okamura, I., Nagahata, H., Murata, R., Mwebembezi, W., Muramatsu, Y. and Makita, K. 2020. Prevalence of sub-clinical mastitis and its association with milking practices in an intensive dairy production region of Uganda. *Journal of Veterinary Medical Science* **82**.

63. Miyasaka, T., Takeshima, S. N., Jimba, M., Matsumoto, Y., Kobayashi, N., Matsuhashi, T., Sentsui, H. and Aida, Y. 2013. Identification of bovine leukocyte antigen class II haplotypes associated with variations in bovine leukemia virus proviral load in Japanese Black cattle. *Tissue Antigens* **81**: 72-82.

64. Murakami, K., Kobayashi, S., Konishi, M., Kameyama, K., Yamamoto, T. and Tsutsui, T. 2011. The recent prevalence of bovine leukemia virus (BLV) infection among Japanese cattle. *Vet Microbiol* **148**: 84-88.

65. Murakami, K., Kobayashi, S., Konishi, M., Kameyama, K. and Tsutsui, T. 2013. Nationwide Survey of Bovine Leukemia Virus Infection among Dairy and Beef Breeding Cattle in Japan from 2009-2011. *Journal of Veterinary Medical Science* **75**: 1123-1126.

66. NAHMS, U. 2014. Dairy 2014: Health and Management Practices in the United States, 2014. https://www.aphis.usda.gov/animal_health/nahms/dairy/downloads/dairy14/Dairy14_dr_P artIII.pdf (Accessed Oct 25, 2020)

67. NAHMS, U. 2014. Dairy 2014: Milk Quality, Milking Procedures, and Mastitis in the United States , 2014. https://www.aphis.usda.gov/animal_health/nahms/dairy/downloads/dairy14/Dairy14_dr_Mastitis.pdf (Accessed Oct 25, 2020)

68. Nakada, S., Kohara, J. and Makita, K. 2018. Estimation of circulating bovine leukemia virus levels using conventional blood cell counts. *Journal of dairy science* **101**: 11229-11236.

69. Nekouei, O., VanLeeuwen, J., Sanchez, J., Kelton, D., Tiwari, A. and Keefe, G. 2015. Herd-level risk factors for infection with bovine leukemia virus in Canadian dairy herds. *Prev Vet Med* **119**: 105-113.

70. Nekouei, O., VanLeeuwen, J., Stryhn, H., Kelton, D. and Keefe, G. 2016. Lifetime effects of infection with bovine leukemia virus on longevity and milk production of dairy cows. *Prev Vet Med* **133**: 1-9.

71. Nikbakht Brujeni, G., Ghorbanpour, R. and Esmailnejad, A. 2016. Association of BoLA-DRB3.2 Alleles with BLV Infection Profiles (Persistent Lymphocytosis/Lymphosarcoma) and Lymphocyte Subsets in Iranian Holstein Cattle. *Biochemical genetics* **54**: 194-207.

72. Nishiike, M., Haoka, M., Doi, T., Kohda, T. and Mukamoto, M. 2016. Development of a preliminary diagnostic measure for bovine leukosis in dairy cows using peripheral white blood cell and lymphocyte counts. *J Vet Med Sci* **78**: 1145-1151.

73. Norby, B., Bartlett, P. C., Byrem, T. M. and Erskine, R. J. 2016. Effect of infection with bovine leukemia virus on milk production in Michigan dairy cows. *Journal of dairy science* **99**: 2043-2052.

74. Notsu, K., Hashida, S., Mitoma, S., Kubo, M., Arikawa, G., Agah, M. A., El-Khaiat, H. M., Mai, T. N., Nguyen, T. H., Elhanafy, E., Daous, H. E., Norimine, J. and Sekiguchi, S. 2018. Assessment of Hematological Parameters and Carcass Weight in Bovine Leukemia Virus Infection in Slaughtered Beef Cattle. *Journal of Veterinary Epidemiology* **22**: 43-48.

75. Nuotio, L., Rusanen, H., Sihvonen, L. and Neuvonen, E. 2003. Eradication of enzootic bovine leukosis from Finland. *Prev. Vet. Med.* **59**: 43-49.

76. Ohno, A., Takeshima, S. N., Matsumoto, Y. and Aida, Y. 2015. Risk factors associated with increased bovine leukemia virus proviral load in infected cattle in Japan from 2012 to 2014. *Virus research* **210**: 283-290.

77. Olde Riekerink, R. G., Barkema, H. W. and Stryhn, H. 2007. The effect of season on somatic cell count and the incidence of clinical mastitis. *Journal of dairy science* **90**: 1704-1715.

78. Otte M.J., Chilonda P. 2000: Animal Health Economics: An Introduction. Animal Production and Healthy Division (AGA), FAO, Rome, Italy. 12 pp http://www.fao.org/ag/againfo/resources/en/publications/agapubs/pproc01.pdf (Accessed Oct 25, 2020)

79. Ott, S. L., Johnson, R. and Wells, S. J. 2003. Association between bovine-leukosis virus seroprevalence and herd-level productivity on US dairy farms. *Preventive Veterinary Medicine* **61**: 249-262.

80. Panei, C. J., Suzuki, K., Echeverría, M. G., Serena, M. S., Metz, G. E. and González, E. T. 2009. Association of BoLA-DRB3. 2 alleles with resistance and susceptibility to persistent lymphocytosis in BLV infected cattle in Argentina. *International Journal of Dairy Science* **4**.

81. Rezac, D. J., Thomson, D. U., Siemens, M. G., Prouty, F. L., Reinhardt, C. D. and Bartle, S. J. 2014. A survey of gross pathologic conditions in cull cows at slaughter in the Great Lakes region of the United States. *Journal of dairy science* **97**: 4227-4235.

82. Robin, X., Turck, N., Hainard, A., Tiberti, N., Lisacek, F., Sanchez, J.-C. and Müller, M. 2011. pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC Bioinformatics* **12**: 77.

83. Rodríguez, S. M., Florins, A., Gillet, N., de Brogniez, A., Sánchez-Alcaraz, M. T., Boxus, M., Boulanger, F., Gutiérrez, G., Trono, K., Alvarez, I., Vagnoni, L. and Willems, L. 2011. Preventive and therapeutic strategies for *bovine leukemia virus*: lessons for HTLV. *Viruses* **3**.

84. Roland, L., Drillich, M. and Iwersen, M. 2014. Hematology as a diagnostic tool in bovine medicine. *Journal of Veterinary Diagnostic Investigation* **26**: 592-598.

85. Rollin, E., Dhuyvetter, K. C. and Overton, M. W. 2015. The cost of clinical mastitis in the first 30 days of lactation: An economic modeling tool. *Prev Vet Med* **122**: 257-264.

86. ROMDHANE, S. and BELKACEM, L. Frailty Modeling for clustered survival data: a simulation study, ed.

87. Rondeau, V., Filleul, L. and Joly, P. 2006. Nested frailty models using maximum penalized likelihood estimation. *Statistics in Medicine* **25**: 4036-4052.

88. Ruggiero, V. J., Norby, B., Benitez, O. J., Hutchinson, H., Sporer, K. R. B., Droscha, C., Swenson, C. L. and Bartlett, P. C. 2019. Controlling bovine leukemia virus in dairy herds by identifying and removing cows with the highest proviral load and lymphocyte counts. Journal of dairy science 102: 9165-9175.

89. Sajiki, Y., Konnai, S., Okagawa, T., Nishimori, A., Maekawa, N., Goto, S., Watari, K., Minato, E., Kobayashi, A. and Kohara, J. 2019. Prostaglandin E2–Induced Immune Exhaustion and Enhancement of Antiviral Effects by Anti–PD-L1 Antibody Combined with COX-2 Inhibitor in Bovine Leukemia Virus Infection. *The Journal of Immunology* **203**: 1313-1324.

90. Sakamoto, K., Kanno, T., Yamakawa, M., Yoshida, K., Yamazoe, R. and Murakami, Y. 2002. Isolation of foot-and-mouth disease virus from Japanese black cattle in Miyazaki Prefecture, Japan, 2000. *J. Vet. Med. Sci.* **64**: 91-94.

91. Schisterman, E. F., Perkins, N. J., Liu, A. and Bondell, H. 2005. Optimal cut-point and its corresponding Youden Index to discriminate individuals using pooled blood samples. *Epidemiology (Cambridge, Mass.)* **16**: 73-81

92. Somura, Y., Sugiyama, E., Fujikawa, H. and Murakami, K. 2014. Comparison of the copy numbers of bovine leukemia virus in the lymph nodes of cattle with enzootic bovine leukosis and cattle with latent infection. *Arch Virol* **159**: 2693-2697.

93. Sordillo, L. 2016. Nutritional strategies to optimize dairy cattle immunity. *Journal of dairy science* **99**: 4967-4982.

94. Sorge, U. S., Lissemore, K., Cantin, R. and Kelton, D. F. 2011. Short communication: Milk ELISA status for bovine leukosis virus infection is not associated with milk production in dairy cows. *Journal of dairy science* **94**: 5062-5064.

95. Tajima, S., Takahashi, M., Takeshima, S. N., Konnai, S., Yin, S. A., Watarai, S., Tanaka, Y., Onuma, M., Okada, K. and Aida, Y. 2003. A mutant form of the tax protein of bovine leukemia virus (BLV), with enhanced transactivation activity, increases expression and propagation of BLV in vitro but not in vivo. *Journal of virology* **77**: 1894-1903.

96. Team, R. 2020. RStudio: Integrated Development for R (1.2. 5042)[Computer software]. RStudio, ed., Inc.

97. Team, R. C. 2015. R version 3.2. 2. *R Project for Statistical Computing, Vienna, Austria. www. r-project. org*.

98. Therneau, T. M. 2020. coxme: Mixed Effects Cox Models.

99. Thomas-Bachli, A. L., Pearl, D. L., Friendship, R. M. and Berke, O. 2014. Exploring relationships between whole carcass condemnation abattoir data, non-disease factors and disease outbreaks in swine herds in Ontario (2001-2007). *BMC research notes* **7**: 185.

100. Thompson, D., Muriel, P., Russell, D., Osborne, P., Bromley, A., Rowland, M., Creigh-Tyte, S. and Brown, C. 2002. Economic costs of the foot and mouth disease outbreak in the United Kingdom in 2001. *Revue scientifique et technique (International Office of Epizootics)* **21**: 675-687.

101. Thursfield, M. 2007. Veterinary epidimiology, Third ed., Blackwell Publishing, Oxford, UK.

102. Tiwari, A., Vanleeuwen, J. A., Dohoo, I. R., Keefe, G. P., Haddad, J. P., Tremblay, R., Scott, H. M. and Whiting, T. 2007. Production effects of pathogens causing bovine leukosis, bovine viral diarrhea, paratuberculosis, and neosporosis. *Journal of dairy science* **90**: 659-669.

103. US Department of Agriculture Animal and Plant Health Services, Veterinary Services, Center for Epidemiology and Animal Health. Info sheet: bovine leukosis virus (BLV) on U.S. dairy operations. 2007. Accessed Sep. 5, 2017. http://www.aphis.usda.gov/animal_health/nahms/dairy/downloads/dairy07/Dairy07_is_BLV.pdf.

104. VanderWeele, T. J. 2016. Mediation Analysis: A Practitioner's Guide. *Annual review of public health* **37**: 17-32.

105. Van Eijk, M. J. T., Stewart-Haynes, J. A., Beever, J. E., Fernando, R. L. and Lewin, H. A. 1992. Development of persistent lymphocytosis in cattle is closely associated with DRB2. *Immunogenetics* **37**: 64-68.

106. Ver Hoef, J. M. and Boveng, P. L. 2007. Quasi-Poisson vs. negative binomial regression: how should we model overdispersed count data? *Ecology* **88**: 2766-2772.

107. Watanabe, A., Murakami, H., Kakinuma, S., Murao, K., Ohmae, K., Isobe, N., Akamatsu, H., Seto, T., Hashimura, S., Konda, K., Shinozuka, Y. and Kawai, K. 2019. Association between bovine leukemia virus proviral load and severity of clinical mastitis. *Journal of Veterinary Medical Science*

108. White, T. L. and Moore, D. A. 2009. Reasons for whole carcass condemnations of cattle in the United States and implications for producer education and veterinary intervention. *Journal of the American Veterinary Medical Association* **235**: 937-941.

109. World Organisation for Animal Health. 2012. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. In Enzootic Bovine Leucosis, Chapter 2.4.10. 7th ed. World Organization for Animal Health (OIE), Paris, France. Accessed Sep. 5, 2017. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.04.10_EBL.pdf.

110. World Organisation for Animal Health. 2020. Official disease status. 2020 https://www.oie.int/en/animal-health-in-the-world/official-disease-status/ (Accessed Oct 25, 2020)

111. Yoshikawa, H., Xie, B., Oyamada, T., Hiraga, A. and Yoshikawa, T. 1997. Detection of Bovine Leukemia Viruses (BLV) in Mammary Tissues of BLV Antibody-Positive Cows Affected by Subclinical Mastitis. *Journal of Veterinary Medical Science* **59**: 301-302.

112. Zeryehun, T. and Abera, G. 2017. Prevalence and bacterial isolates of mastitis in dairy farms in selected districts of eastern Harrarghe Zone, Eastern Ethiopia. *Journal of veterinary medicine* **2017**.

Abstract

Application of epidemiology and animal health economics in control of bovine leukemia virus (BLV) infection among dairy cows in Hokkaido Prefecture

Veterinary Epidemiology
Doctoral Course of Veterinary Medicine
Graduate School of Veterinary Medicine
Rakuno Gakuen University Graduate School
Satoshi Nakada

The objective of this thesis is to provide useful information in planning control options of bovine leukemia virus (BLV) infection in dairy cows in Hokkaido Prefecture by using epidemiology and animal health economics. To achieve the objective, three studies were conducted. The first study established a cost-effective and rapid identification method for high proviral load (H-PVL) cows in a herd. The second and third studies showed how damages in immune systems due to BLV affect the productivity, and how much money the dairy industry are losing by allowing H-PVL cows to stay and spread BLV in dairy herds in Japan.

In Chapter 1, establishment of a quantitative procedure for estimating blood PVL/μl using a statistical model was attempted. In the MAFF's guideline for BLV control, the "test and segregate" or "test and manage" strategy is recommended, and diagnostic methodologies such as ELISA, qualitative PCR, and quantitative real-time PCR were introduced. However, they can be costly in testing large number of cows. In Europe, the EC 's key was used as an indicator to cull potentially BLV-infected cows and eradication of BLV has been achieved. The EC 's key uses the age and lymphocyte count of cows. My model further improved the EC's key because it estimates BLV PVL using these information. Priority culling of H-PVL cows has been

reported to reduce BLV transmission as well as prevalence efficiently. My model does not require expensive measurement equipment and allow identifying potential H-PVL cows for priority culling.

In Chapter 2, the economic loss due to BLV-associated carcass weight reduction of dairy cows was estimated. Dairy cows are an important source of beef, and quantification of such economic loss is important in understanding the impact of BLV. The number of EBL cattle condemned at meat inspection in slaughterhouses has been increasing in Japan due to the increase in BLV prevalence, hence the economic importance is increasing. A hypothesis that decreased immune function due to BLV infection would increase visceral abnormalities, in turn reducing carcass weight was formulated, and was tested using mediation analysis. As a result, this study revealed that a mean carcass weight for H-PVL culled cows was significantly lighter, by 30.2 kg than that of non-infected cows. When the number of abnormal findings postmortem examination (AFPE) is increased by 1, carcass weight significantly decreased by 8.55 kg. The mediation analysis indicated that the indirect effect was significant for H-PVL culled cows, whereas not significant for L-PVL. The proportion of the effect mediated by AFPE was 21.6% for H-PVL culled cows, a value that was much larger than the 8.7% obtained for L-PVL culled cows. The median annual direct loss of carcass weight due to BLV infection in Hokkaido Prefecture in 2017 was estimated to be 118,166 kg, and the annual economic loss was 66,172,680 yen, which was 626,277 US dollars.

In Chapter 3, the economic loss due to BLV- associated mastitis in Hokkaido Prefecture was estimated. Mastitis is a major economic constraint in dairy industry, and 72.8% of cows with mastitis recover and remain in the herd, while 24% are removed or sold. In 2017, there were about 265,000 cases of clinical mastitis in Hokkaido. The hypothesis in this study was that suppressed immune function due to BLV infection would increase the incidence of mastitis and was tested using survival analysis. The hazard ratio for mastitis for H-PVL cows was 2.6 times higher than that of BLV-non-infected cows. The Kaplan–Meier survivor curves proved that a

half of H-PVL cows suffered from mastitis within 52 days after calving, and among the mastitis observed within 310 days since delivery, a half occur within 30 days. The economic loss due to mastitis associated with BLV infection per H-PVL cow was 43,085 yen and 416 US dollars, and the annual economic loss due to mastitis caused by BLV infection in Hokkaido Prefecture was estimated to be 122,351,721 yen, which is 1,182,142 US dollars. Overall, my study revealed the high economic loss due to BLV infection on the dairy production in Hokkaido Prefecture.

This thesis provided enough information to convince the dairy industry and the government to accelerate the policy development and voluntary efforts in BLV control. The approaches described in this thesis should be well applicable to solve problems of the dairy industry in Hokkaido, Japan, including other important diseases such as Johne’s disease.

Abstract in Japanese (和文要旨)

北海道の酪農における牛白血病ウイルス感染制御のための疫学および家畜衛生経済学の応用に関する研究

酪農学園大学大学院獣医学研究科
獣医学専攻博士課程
獣医疫学　中田悟史

本研究論文の目的は、疫学および家畜衛生経済学を用いて、北海道の乳用牛における牛白血病ウイルス(BLV)の制御に有用な情報を提供することである。目的を達成するために、3つの研究を行った。最初の研究は、牛群中の BLV 高ウイルス血症牛(H-PVL 牛)を安価で迅速に同定する方法を確立した。第 2 と第 3 の研究は、BLV による免疫不全が生産性にどのような影響を与えるか、また、日本の乳用牛群に H-PVL 牛を在籍させ、BLV を蔓延させることで酪農業にどれ程の損失を与えているかを明らかにした。

第 1 章では、統計モデルを用いた血中ウイルス量(PVL/μl)の定量的な推定方法の確立を試みた。農水省の BLV 管理ガイドラインでは、「検査と分離」または「検査と管理」が推奨されており、ELISA、定性的 PCR、定量的リアルタイム PCR などの診断法が紹介されている。しかし、これらの方法は大量の牛を検査するにはコストが嵩む。ヨーロッパでは EC key が BLV に感染した可能性のある牛を淘汰するための指標となり、BLV の根絶が達成された。EC key は牛の年齢とリンパ球数を用いている。本モデルは、これらの情報を用いて BLV PVL を推定すべく、EC key をさらに改良した。H-PVL 牛の優先的な淘汰は、BLV 感染を効率的に減少させることが報告されている。本モデルは高価な測定機器を必要とせず、優先淘汰の対象となる 潜在的 H-PVL 牛を特定することを可能にした。

第 2 章では、BLV に伴う乳牛の枝肉重量減少による経済的損失を試算した。

乳牛は牛肉の重要な供給源であり、その経済損失を定量化することは BLV の影響を理解する上で重要である。日本では BLV 感染率の増加に伴い、食肉処理場の食肉衛生検査で摘発される牛リンパ腫発症牛が増加しており、経済的重要性が高まっている。そこで、BLV 感染による免疫機能の低下が内臓異常を増加させ、枝肉重量を減少させるという仮説を立て、媒介分析を用いて検証した。その結果、H-PVL と畜牛の平均枝肉重量は、非感染と畜牛に比べて 30.2kg 有意に軽くなった。と畜時の内臓異常所見（AFPE ）が 1 増加すると、枝肉重量は 8.55kg 有意な減少を示した。媒介分析の結果、間接効果は H-PVL では有意であったが、L-PVL では有意ではなかった。AFPE を媒介した効果の割合は H-PVL では 21.6%で、L-PVL では 8.7%であり大きく上回った。2017 年の北海道における BLV 感染による枝肉重量の直接的な損失額の中央値は年間 118,166kg、経済的損失額は年間 66,172,680 円で、626,277 ドルと推定された。

第 3 章では、北海道の BLV に関連する乳房炎による経済損失を試算した。乳房炎は酪農業の経済的な大きな制約であり、乳房炎発症牛の 72.8%は回復して牛群に在籍するが、24%が淘汰や売却されている。2017 年の北海道での臨床型乳房炎発症牛約 26 万 5000 頭であった。本研究では、BLV 感染による免疫機能の抑制が乳房炎の発症率を高めるという仮説を立て、生存分析を用いて検証した。その結果、H-PVL の乳房炎のハザード比は BLV 非感染牛の 2.6 倍であった。Kaplan-Meier 生存曲線から、H-PVL の半数が分娩後 52 日以内に乳房炎を発症しており、分娩後 310 日以内に発症した乳房炎のうち、半数が 30 日以内に発症していることが判明した。H-PVL 牛 1 頭当たりの BLV 感染による乳房炎による経済的損失は 43,085 円、416 ドルであり、北海道の BLV 感染による乳房炎による年間経済的損失は 122,351,721 円、1,182,142 ドルと推定された。以上のことから、本研究の結果、北海道の乳生産における BLV 感染症による経済損失の大きさが明らかになった。

本論文は、酪農産業と政府が BLV 対策のための政策展開と自主的な取り組みを加速させるための十分な情報を提供した。本論文に記載されたアプローチは、ヨーネ病のような他の重要な病気を含め、北海道の酪農業の問題を解決するために十分適用可能であると考えられる。